

**Novel Technologies for the diagnosis and  
treatment of high risk patients with Barrett's  
Oesophagus**

**PhD Thesis**

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***“In order to shake a hypothesis, it is  
sometimes not necessary to do  
anything more than push it as far as  
it will go”***

Denis Diderot 1753  
“On the interpretation of Nature”

# **ABSTRACT**

## Abstract

The incidence of oesophageal adenocarcinoma is rising rapidly in the UK with most, if not all, adenocarcinomas arising within Barrett's oesophagus (BO). Defining those patients at high risk is difficult because the histological diagnosis of high grade dysplasia (HGD) is imprecise. Surveillance is unlikely to prove to be of benefit to patients in its current form. Detection of aneuploidy may offer more accurate detection of patients at high risk but its measurement is not currently possible in routine clinical service. Until recently if a patient had HGD the only widely available option was oesophagectomy with all of its adherent risks.

This thesis demonstrated that image cytometry can reliably diagnose aneuploidy, that this is correlated with histology and is accurate compared to flow cytometry performed in Seattle. Additionally, aneuploidy measured by image cytometry was validated in a case-control study of future risk of relapse following ablative therapy in BO.

Elastic scattering spectroscopy (ESS) is a real-time *in vivo* technique sensitive to changes in the physical properties of tissue. This data demonstrated that ESS can reliably detect both HGD and aneuploidy in vivo in BO in algorithm generation and prospective testing. A proof of concept study also suggested that ESS could potentially be used for surveillance.

Photofrin-PDT has led the way in minimally invasive therapy for HGD in BO. The best regimen of ALA-PDT identified in this thesis is 60mg/kg of ALA activated by 1000J/cm of red light. The early data from the RCT of ALA versus Photofrin-PDT appears to show that ALA may be a lower risk alternative with possibly a higher efficacy for the eradication of HGD.

In summary these studies potentially offer the more accurate definition of high risk patients in BO and a reduction in the frequency of endoscopies for those at low risk. Additionally, it has advanced the understanding of ALA-PDT.

## **STATEMENT OF ORIGINALITY**

I, Gary Mackenzie, confirm that the work presented in this thesis is my own. Any contributions from others expressly acknowledged or cited. Specifically the novel advanced statistical techniques relating to experimental error reduction and simultaneous multiple outcome discrimination were developed by Ying Zhu. In Chapter 8 sections 2 and 3 any patients that received PDT before October 2004 were treated by my predecessor, Dr Cheliah Selvasekar.

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## LIST OF ABBREVIATIONS

4D-ELF	4 Dimension Elastic Light-Scattering Fingerprinting
AFI	Autofluorescence Imaging
AGA	American Gastroenterological Association
ALA	Aminolaevulinic Acid
APC	Argon Plasma Coagulation
BMI	Body Mass Index
BO	Barrett's Oesophagus
Bootstrap	Block validation statistical analysis with a proportion of the dataset assigned for training and testing (eg 70:30 ratio)
BSG	British Society of Gastroenterology
Buried Glands	Barrett's underneath re-epithialised squamous mucosa
CI	Confidence Interval
CLO	Columnar-lined Oesophagus
COREC	Central Office of Research Ethics Committees
COX2	Cyclo-oxygenase 2
DNA Index	Mean IOD of the whole image cytometry histogram divided by the IOD of 2N (a diploid nucleus)
DIF	Drug Induced Fluorescence
EMR	Endoscopic Mucosal Resection
ESACP	European Society of Analytical Cellular Pathology
ESS	Elastic Scattering Spectroscopy
EU	European Union
EUS	Endoscopic Ultrasound
FCM	Flow cytometry
GORD	Gastro-oesophageal Reflux Disease
HCl	Hydrochloric Acid
HGD	High Grade Dysplasia
HR	Hazard Ratio
ICM	Image cytometry
IOD	Integrated Optical Density
ISRCTN	International Standard Randomised Controlled Trial Number
Jackknife	Leave one out cross validation statistical analysis
LDA	Linear Discriminant Analysis
LGD	Low Grade Dysplasia
LIFE	Light Induced Fluorescence Endoscopy
LOH	Loss of Heterozygosity
LOS	Lower Oesophageal Sphincter
LSS	Light Scattering Spectroscopy
MBDB	Methylene Blue Directed Biopsies
MHRA	Medicine and Healthcare products Regulatory Authority
MPEC	Multipolar electrocoagulation
mTHPC	meta-Tetrahydroxyphenyl Chlorin
NBI	Narrow Band Imaging
NICE	National Institute for Clinical Excellence
NPV	Negative Predictive Value



OA	Oesophageal Adenocarcinoma
OCT	Optical Coherence Tomography
OGD	Oesophago-Gastro-Duodenoscopy
OR	Odds Ratio
PBS	Phosphate Buffered Saline
PC	Principle Component
PCA	Principle Component Analysis
PDT	Photodynamic Therapy
PET	Positive Emission Tomography
Photofrin	Porfimer Sodium
PPI	Proton Pump Inhibitor
PPIX	Protoporphyrin IX
PPV	Positive Predictive Value
QALY	Quality Adjusted Life Year
RFA	Radiofrequency Ablation
RR	Relative Risk
SAE	Serious Adverse Event
SIM	Specialised Intestinal Metaplasia
SmPC	Summary of Product Characteristics
UCH	University College Hospital

# Introduction

Barrett's Oesophagus: Novel  
Diagnostic and Therapeutic  
Techniques

## Chapter 1

# Development of Barrett's Oesophagus, Surveillance and Risk Factors for Cancer

## **1.1 Adenocarcinoma of the Oesophagus**

The incidence of oesophageal adenocarcinoma is rapidly rising with rates in the UK higher than those in the USA. Scotland has the highest incidence in the World (Lagergren, 2005). It is now the 4<sup>th</sup> commonest cause of cancer related mortality in the UK in men and the 7<sup>th</sup> in women (UK National Statistics, 2007). By the time patients develop symptoms there are radiologically visible metastasis in 50% of patients (Enzinger and Mayer, 2003) and this translates to an appalling overall five year survival rate of only 5-7% with current management (Newnham et al, 2003). Even in those patients who present with operable cancer the five year survival following surgery is probably only 15-25% (De Vita et al, 2002; Fernandez and Meyers, 2004; Keighley, 2003; Kelsen et al, 2007). The risks of oesophagectomy are substantial with many units reporting a 4-7.5% mortality rate although some units report a much lower mortality rate for early stage disease. There is also a major morbidity rate of 40% even in high volume centres where the mortality rates are thought to be lower (Dimick et al, 2003; Dimick et al, 2005; Fernandez and Meyers, 2004; Hulscher et al, 2001; Keighley, 2003; Patti et al, 1998; Portale et al, 2006b; Sihvo et al, 2004; Williams et al, 2007). A recent single centre study of 283 patients has reported a higher 5 year survival rate at 46% but the complication rate was also higher at 60% (Portale et al, 2006a). This may reflect more accurate cancer staging, the use of neoadjuvant chemotherapy and more careful patient selection prior to resection. Furthermore, the quality of life of patients undergoing either transhiatal or transthoracic resection did not return to baseline for up to a year after surgery (De Boer et al, 2004).

This poor five year survival and advanced nature of the disease at presentation has led clinicians to focus on surveillance in the premalignant condition, Barrett's Oesophagus (BO).

## **1.2 Definitions of Barrett's Oesophagus**

Barrett's Oesophagus was first described by Norman Barrett in the 1950s but has lacked a widely accepted definition. One definition suggested by Spechler in 1992 was: *'the replacement of the normal squamous epithelial lining of the oesophagus with specialised intestinal metaplasia which is more resistant to damage from gastric contents'* (Spechler, 2002). This definition, however, does not prevent potential confusion as to where this 'gastro-oesophageal junction' starts with the diagnosis being dependent on endoscopic recognition of this mucosal change and a subjective

judgement as to its precise location. Attempts have been made to define this to reduce interobserver variation:

*'The proximal limit of linear gastric mucosal folds is the most practicable indicator of the gastro-oesophageal junction in the presence of suspected Barrett's oesophagus in routine diagnostic endoscopic practice'* (Armstrong, 2004).

This leaves one further problem and that is the definition of the exact limit of the gastric folds: *'The proximal limit of gastric mucosal folds is defined best as the most proximal point at which there is any evidence of a linear fold of gastric mucosa. This is best visualized when the oesophagus is distended minimally to the point at which the proximal ends of the gastric folds appear'* (Armstrong, 2004).

Recently, the two former definitions were considered by a multidisciplinary international workshop comprising 18 experts under the auspices of the American Gastroenterological Association (AGA). One of the tasks of this group was to try to form a consensus on the evidence to support some fundamental definitions in Barrett's. The statement "The proximal margin of the gastric folds is a reliable endoscopic marker for the gastroesophageal junction" was accepted (with reservations) by all members of the workshop and is also recommended by the 2005 BSG guidelines (Sharma et al, 2004).

"Oesophageal intestinal metaplasia documented by histology is a prerequisite criterion for the diagnosis of Barrett's Oesophagus". This statement was accepted albeit with reservation by all but two of the international panel. To add further difficulty and make study comparison more complex the British definition of Barrett's Oesophagus (or perhaps less confusingly columnar-lined oesophagus, CLO) which is supported by the recent BSG guidelines suggested that specialised intestinal metaplasia (SIM) is not required for the diagnosis of Barrett's CLO (Watson et al, 2005). The feeling is that specialised intestinal metaplasia may be present but just 'missed' by the sampling error of random biopsies. This is the prevailing opinion in the UK. One recent UK study supports CLO in the absence of SIM be included in the Barrett's population. This study demonstrated no significant difference in the future cancer risk of 712 patients followed up for a median of 12 years irrespective of the presence of SIM. The adenocarcinoma rates were 4.5% (17/379) in the group with SIM and 3.6% (11/309) in the group with CLO only (Kelty et al, 2007). This disagreement is unlikely to be settled soon and therefore studies between countries will continue to have different enrolment criteria.

### **1.3 Risk factors for developing Barrett's Oesophagus and Oesophageal Adenocarcinoma**

Barrett's Oesophagus is thought to result from longstanding GORD (Spechler and Goyal, 1986) and BO in turn is a premalignant condition that predisposes to the development of oesophageal adenocarcinoma by 30-125 times (Cameron et al, 1985; Hameeteman et al, 1989; Robertson et al, 1988; Williamson et al, 1991). See table 1.1. The risk of adenocarcinoma also appears to be strongly related to the severity in addition to the duration of acid exposure (Avidan et al, 2002; Lagergren et al, 1999a; Ye et al, 2001). Additionally, the presence of a hiatus hernia appears to increase the risk of cancer development proportional to its length (Avidan et al, 2002; Weston et al, 1999) as does the presence of oesophageal injury, oesophagitis (Fein, 1997; Ye et al, 2001) and alkaline biliary reflux as measured by Bilitec monitoring (Fein et al, 1997). Finally the UK Barrett's Oesophagus Registry has published data regarding the length of Barrett's and future cancer risk. In 1000 patients from 5 different hospitals 28 patients developed cancer with higher cancer rates seen in short segments <3cm and long segment >6cm at 5.8% and 7.1% respectively when compared to Barrett's 3-6cm in length with a risk of 1.6% (Gatenby et al, 2003).

There are no reliable estimates of the prevalence of BO in the population. It is generally accepted that most, if not all, adenocarcinomas arise from Barrett's oesophagus (Conio and Cameron, 2001; Kim et al, 1997) and approximately 40% of oesophageal cancers occur without symptoms of reflux (Gerson et al, 2002) with only 5% having a prior diagnosis of BO (Conio et al, 2001; Dulai et al, 2002). Attempts have been made to estimate the undiagnosed proportion of patients with BO in the general population. Several studies have examined the prevalence of BO in patients undergoing an endoscopy for GORD and estimated it to be between 10% and 16% (Koop, 2000; Westhoff et al, 2005; Winters, Jr. et al, 1987). In a further study 961 patients undergoing colonoscopy were offered a gastroscopy. Of these 556 had a history of heartburn and 384 were symptom free. The prevalence of BO was 8.3% and 6.3% respectively (Rex et al, 2003). In another cohort of 110 patients over the age of 50 who were having surveillance sigmoidoscopy for colorectal cancer the prevalence of BO was 25%. (Gerson et al, 2002). Finally, an autopsy study has shown that a very large number of cases of BO remain undiagnosed in life with autopsy diagnosing over a twenty times increase in the prevalence of Barrett's (Cameron and Carpenter, 1997). It is now considered that with increased availability of endoscopic procedures this figure may have risen to 20% of cases of BO being diagnosed clinically (Conio et al, 2001).

**Table 1.1: Risk factors for the development of Barrett's Oesophagus**

<b>Risk Factor</b>	<b>Selected References</b>	<b>RR or OR For BO</b>	<b>RR or OR For Cancer</b>	<b>Comments</b>
GORD	Spechler 1986, Lagergren 1999, Ye 2001, Anderson 2007, Avidan 2002.	9 (5.7-14.1)	7.7 (5.3-11.4)	Severity and Chronicity important
Alkaline Biliary Reflux	Fein 1997	4.2	Increased exposure 30% vs 7%	
Hiatus Hernia >4cm	Avidan 2002, Weston 1999.	4.1	Approx. 2.0	
Sex	Caygill 2004, Jankowski 2005, Blot 1991, Atkinson 1992, Hansson 1993, Avidan 2002.	2:1 Male: Female	3:1 to 8:1	
Obesity	Kubo 2006, MacInnis 2006. Anderson 2007	Up to 3.7	Meta-analysis 2.4	BMI>30 cf Normal
Smoking	Anderson 2007 Vaughan 1998, Avidan 2002	Possible but no consistent association in studies		Studies vary
Drugs Relaxing LOS	Lagergren 2000, Vaughan 1998.	No data	Up to 3.8	May be confounded by reflux
Alcohol Consumption	Lagergren 2000, Avidan 2002, Gammon 1997	Unlikely	Unlikely	Limited to those with family history
Family History	Chak 2002 & 2004, Romero 1997	2x		
Ethnic origin	Blot 1991, Avidan 2002	Up to 22 for Caucasians		Paucity of evidence in non Caucasians

RR=Relative Risk, OR=Odds Ratio

There is a male predominance in the prevalence of Barrett's oesophagus with a Male: Female ratio of approximately 2:1 (Caygill et al, 2004; Jankowski, 2005). Furthermore, there is an increased incidence of oesophageal adenocarcinoma amongst men with a male:female ratio between 3 and 8:1 (Atkinson et al, 1992; Avidan et al, 2002; Blot et al, 1991; Hansson et al, 1993). Adenocarcinoma also appears to occur more commonly in Caucasians than other ethnic groups (Avidan et al, 2002; Blot et al, 1991) .

There have been three large population based studies and one recent meta-analysis which have suggested that an increased Body Mass Index (BMI) is associated with both an increased risk of BO and oesophageal adenocarcinoma (Chow et al, 1998; Gammon et al, 1997; Kubo and Corley, 2006; Lagergren et al, 1999b; MacInnis et al, 2006). The meta-analysis suggested an increased

risk amongst obese individuals with an odds ratio of 2.4 (Kubo and Corley, 2006) which was broadly similar to the largest of the cohort studies of 41,295 people which reported a hazard ratio of 3.7 for obese people ( $\text{BMI} > 30 \text{ kg/m}^2$ ) compared to those with a normal BMI. (MacInnis et al, 2006) One study has suggested that this increased risk may be due to increased reflux amongst this obese group (Kouklakis et al, 2005).

There are mixed results amongst the studies examining the effect of smoking on future cancer risk. Some studies report an increased risk with an odds ratio up to 2.8 (Anderson et al, 2007; Eisen et al, 1997; Gammon et al, 1997; Levi et al, 1990; Wu et al, 2001) while others report no higher risk in either univariate or multivariate analysis (Avidan et al, 2002; Bani-Hani et al, 2005; Vaughan et al, 1998).

Medications that relax the lower oesophageal sphincter have also been implicated in carcinogenesis such as nitrates, benzodiazepines, anticholinergics, theophyllines and calcium channel blockers. One study suggested an increased OR of 3.8 amongst daily long term (>5years) users. This risk corrects to baseline with adjustment for reflux suggesting this as the possible method of action. Furthermore, the authors estimated the number of patients needed to be treated for resultant harm to be 5570 for one case of adenocarcinoma in those over the age of 60 (Lagergren et al, 2000a). A second study reported an odds ratio of 3.1 for the development of cancer with long term (>5year) theophylline use although this study did not show an increased risk with other drugs (Vaughan et al, 1998).

Alcohol consumption appears to have limited or no impact on cancer risk (Avidan et al, 2002; Gammon et al, 1997; Lagergren et al, 2000b). There have been reports of clustering of cases in families as well as increased risk of Barrett's amongst family members of patients with either existing Barrett's or adenocarcinoma (Chak et al, 2002; Chak et al, 2004; Romero et al, 1997). The effect of inherited genetics on the overall population with Barrett's and the development of adenocarcinoma, however, appeared limited with many environmental factors of apparent greater importance. Finally, for the first time the FINBAR study examined the importance of fruit and vegetables in the diet of those with BO and associated these dietary factors with future cancer risk. The FINBAR study is an Irish population based case control study which recruited 224 patients with BO and 227 patients who developed oesophageal adenocarcinoma. This group found that smoking, GORD and raised BMI all increased a patient's risk of oesophageal cancer but a high dietary fruit intake was protective (OR 0.50) (Anderson et al, 2007).



Despite the described risk factors for both the development of BO and oesophageal adenocarcinoma clinicians are still unable to accurately predict those patients with BO on clinical criteria. This causes particular problems with screening for Barrett's in the population using endoscopy as large numbers of negative endoscopies are unacceptable to patients and also unacceptable on the grounds of cost. Cheaper and more acceptable methods other than endoscopy would be necessary were screening to be seriously considered with this method probably targeted to those patients at least over 50 years old and also need to be directed at males with some history of acid reflux.

#### **1.4 Metaplasia-Dysplasia-Carcinoma Sequence**

There is convincing evidence that Barrett's oesophagus progresses to cancer via a metaplasia-dysplasia-carcinoma sequence (Haggitt, 1994; Montgomery et al, 2001b; Rajan et al, 2001; Spechler, 1997; Weston et al, 1999). Furthermore it has been suggested that Barrett's oesophagus confers an annual risk of developing adenocarcinoma between 0.2 and 2% which translates to an approximate 5% lifetime risk (Jankowski et al, 2002; Shaheen, 2001; Spechler, 2000). Until 2000 the traditional Japanese and Western classification systems for epithelial neoplasia of the gastrointestinal tract had led to large differences in reporting between pathologists. A unified method of reporting was subsequently agreed at an international symposium which increased agreement from 14% to 62% for oesophageal lesions (Schlempler et al, 2000).

The agreed "Vienna classification" system devised was:

1. Negative for dysplasia,
2. Indefinite for dysplasia,
3. Low grade dysplasia,
4. High grade dysplasia (including non-invasive carcinoma and suspicion of invasive carcinoma),
5. Invasive carcinoma.

(Schlempler et al, 2000)

A more recent and potentially more accurate estimate because of its larger sample size has been suggested by a multicentre cohort study of 618 patients with BO. Ninety-one had been excluded (6.7%) as they were felt to have prevalent cancers defined by diagnosis within 12 months of the patient's index endoscopy. Of the 618 patients studied (mean follow up 4.1 years) the combined

incidence of a histological diagnosis of high grade dysplasia (HGD) or cancer was 1.3% per year. Of these 34 patients eighteen (53%) progressed directly from non-dysplastic Barrett's oesophagus (defined as at least 2 endoscopies with non-dysplastic biopsies) within a three year surveillance interval. In the 156 patients with low grade dysplasia (LGD), progression to HGD or cancer occurred at a rate of 0.6% per year (Sharma et al, 2006).

There have been many studies trying to define these patients' future risk based on dysplasia. Although the results of these studies all suggest significantly increased risk of cancer with HGD the rates of 5 year cancer development vary widely between 13-59% (Montgomery et al, 2001b; Rabinovitch et al, 2001; Schnell et al, 2001; Spechler et al, 2001). Meaningful interpretation of these studies is complicated further by the differences between study populations as well as the difficulty with interobserver variability in the histopathological diagnosis of dysplasia (Fitzgerald and Triadafilopoulos, 1998; Lovat et al, 2006; Montgomery et al, 2001a; Reid et al, 1988). Perhaps the most accurate estimation of future cancer risk comes from the control arm of the randomised controlled trial comparing surveillance with PPI use to Photofrin photodynamic therapy. In the control arm the five year cancer risk was approximately 50% (Overholt et al, 2007).

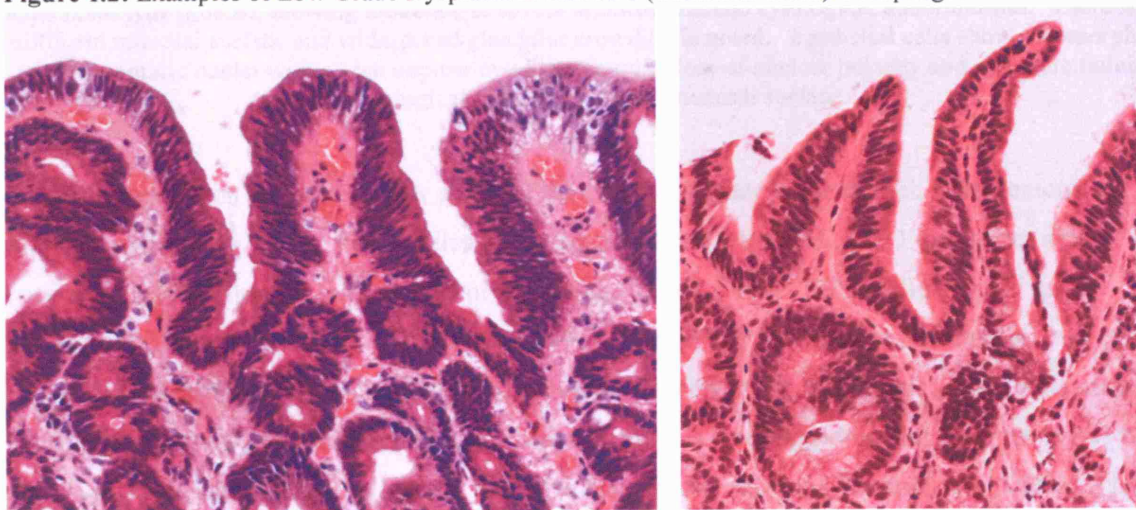
Several studies, however, have suggested that a large number of patients progress directly from no dysplasia or LGD to cancer development without HGD being detected during surveillance. Sharma et al, suggest that this might be as many as 53% of patients and two independent studies concur suggesting approximately 40% of cases progress to cancer without HGD being detected (Reid et al, 1992; Schnell et al, 2001). Failing to accurately define a high risk group in any surveillance program, with up to 50% of patients going on to develop cancer being missed, poses significant difficulties for effectiveness. Unfortunately, this group of patients directly progressing to cancer accounts for only a small percentage (0.6%-8%) of all patients diagnosed as having no dysplasia or only LGD (Reid et al, 2000b; Sampliner, 2002; Schnell et al, 2001; Sharma et al, 2006; Weston et al, 1999). Intensive surveillance or treatment to this entire group is unlikely to be cost effective which therefore poses significant problems for surveillance using dysplasia alone as the marker of future cancer risk.

**Figure 1.1:** Example of histology showing non-dysplastic Barrett's (Columnar-lined) Oesophagus



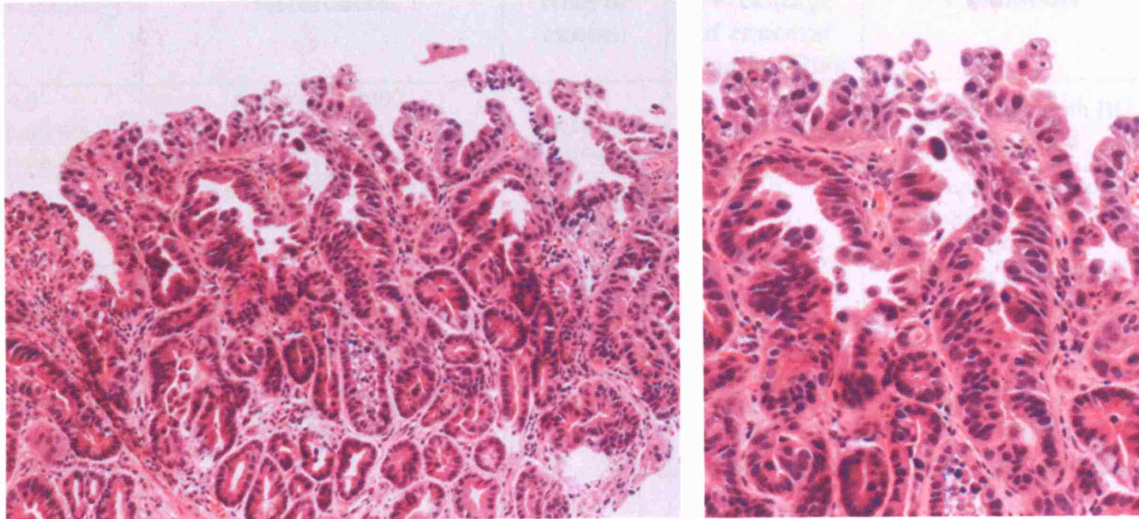
Glandular type mucosa with intestinal metaplasia and mild chronic inflammation. Epithelial nuclei are uniform with low nuclear to cytoplasmic ratios and there is evidence of surface maturation.

**Figure 1.2:** Examples of Low Grade Dysplasia in Barrett's (Columnar-lined) Oesophagus



Glandular type mucosa showing a villiform surface, a mild to moderate increase in nuclear to cytoplasmic ratio and loss of nuclear polarity with nuclear stratification. There is little, if any, evidence of cellular maturation on the mucosal surface.

**Figure 1.3:** Examples of High Grade Dysplasia in Barrett's (Columnar-lined) Oesophagus



Glandular type mucosa showing moderate to severe architectural and cytological abnormalities. There is a villiform mucosal surface and widespread glandular crowding is noted. Epithelial cells show pleomorphic, hyperchromatic nuclei with a high nuclear cytoplasmic ratio, loss of nuclear polarity and complete failure of cytological maturation on the mucosal surface.

Attempts have been made to further quantify this risk with other features such as the extent and diffuse nature of the HGD. Two studies have suggested that with focal HGD the cancer risk is between 17-27% whereas with diffuse or extensive HGD it is up to 56% (follow up 17 and 35 months) (Buttar et al, 2001; Weston et al, 1999). One concern with these two studies, however, is that the results almost certainly included prevalent cancers at the time of inclusion. This may explain the high cancer incidence. A more recent study has also suggested that multiple levels of HGD are associated with the increased risk of metachronous cancers (Tharavej et al, 2006). The presence of other features, such as severe oesophagitis, ulceration, strictures and nodularity, in a Barrett's segment with or without HGD also appears to increase the chances of either future cancer development or it already being present (Bani-Hani et al, 2005; Hillman et al, 2003; Montgomery et al, 2002; Tharavej et al, 2006).

**Table 1.2: Risk of developing cancer with different degrees of dysplasia**

Histology	References	Risk of cancer	Percentage of cancers/ Relative Risk	Comments
All patients with BO	Jankowski 2002, Shaheen, 2001, Spechler, 2000.	0.2-2%/year, 5% lifetime	40-53%	Most patients with BO undiagnosed
LGD	Reid 2000, Schnell 2001, Sharma 2006, Sampliner 2002, Weston 1999	0.6-8% at 5 yrs		Increases with more pathologists agreeing LGD
HGD: All	Montgomery 2001, Overholt 2007, Rabinovitch 2001, Schnell 2001, Spechler 2001	13-56% at 5 yrs	47-60%	Difficult to compare studies. Definitions of HGD
Focal	Weston 1999, Buttar 2001, Tharavej 2006	17-27% at 1½yrs	Diffuse 3.7x RR	Included prevalent cancers
Diffuse		Up to 56% at 3yrs		
Strictures, Nodules, Ulceration	Tharavej 2006, Montgomery 2002, Bani-Hani 2005, Hillman 2003	Double risk of occult Ca	Nodules RR 4x	

The likelihood of detecting dysplasia is associated with the number of biopsies taken (Van Sandick et al, 1998) partly because HGD is patchy and has a mosaic distribution (Reid et al, 1988). Sampling error is hardly surprising because the estimated average area of the Barrett's mucosa with HGD is 1.3 cm<sup>2</sup> out of 32 cm<sup>2</sup> (Cameron and Carpenter, 1997). Even the most rigorous biopsy protocols including those using jumbo biopsy forceps survey less than 1% of the oesophageal mucosa and still miss up to one third of cases with HGD or early cancer (Falk et al, 1999; Kara et al, 2005b; Kara et al, 2005a; Reid et al, 2000a). The Seattle group suggest quadrantic biopsies every centimetre in patients with known HGD in order to detect metachronous cancers which increases the detection rate by 50% (Levine et al, 2000). Taking quadrantic biopsies every two centimetres however is time consuming (Messmann et al, 1999) without doubling the burden by taking biopsies every centimetre.

This considerable albeit inexact increased cancer risk with HGD has driven clinicians to perform endoscopic surveillance with systematic but essentially random biopsies in an attempt to define a high risk group for further therapy or intense surveillance. This method of defining risk, however, is imprecise as demonstrated by the relatively large number of patients who develop cancer either directly from non-dysplastic BO or from low grade dysplasia. A method to survey the Barrett's segment more comprehensively might pick up previously 'missed' areas of HGD in those who go directly from no HGD to cancer. To survey the entire population with BO intensely would appear

to be impractical because the overall progression rate to cancer is low and most patients will never develop it (Jankowski et al, 2002; Reid et al, 2000b; Sharma et al, 2006; Spechler, 2000).

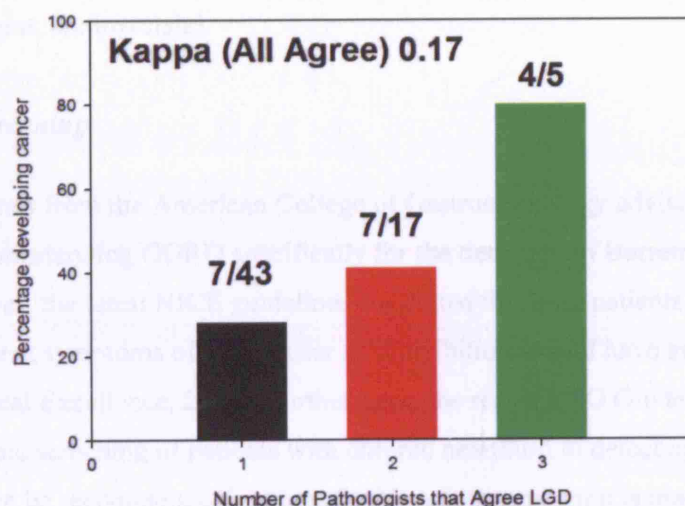
#### ***1.4.1 Inter-observer variability in the diagnosis of dysplasia***

The diagnosis of dysplasia in Barrett's oesophagus is subjective (Fitzgerald and Triadafilopoulos, 1998). The subtle, progressive nature of the changes which occur in Barrett's mucosa from non-dysplastic via low and high grade dysplasia to invasive cancer leads to wide intra and inter-observer variations (Flejou, 2005). Even among the specialist pathologists the agreement on diagnosis of all grades of dysplasia is at best only 50% and they agree on the diagnosis of HGD only 80% of the time (Kerkhof et al, 2007; Lovat et al, 2006; Montgomery et al, 2001a; Reid et al, 1988). In a study performed on the histological assessment by community pathologists in the USA only 30% identified HGD in BO correctly. Furthermore, non dysplastic BO was identified as LGD by 35% and as carcinoma by 5% (Alikhan et al, 1999).

As already stated it has been shown that LGD poorly predicts progression to HGD or cancer and the interobserver variability between histopathologists is high. In an interesting study of the progression of LGD to neoplasia (HGD and cancer), Skacel et al reported that as increasing numbers of histopathologists agree on a diagnosis of LGD future neoplasia risk is increased. Seven out of 43 patients developed neoplasia with a single pathologist reporting LGD, if 2 agreed the risk rose to 41% (7/17) and if all three agreed there was an 80% risk (4/5) (Skacel et al, 2000). See figure 1.4 below.



**Figure 1.4: Agreement between pathologists for LGD determines future cancer risk**



The statistical test for the agreement of all three pathologists, represented by the Kappa Score, was 0.17 suggesting only poor overall pathologist agreement for the diagnosis of LGD.

(Skacel et al, 2000)

It is impractical to expect 3 pathologists to report all slides given the availability of pathologists and cost of doing this in routine clinical practice. All the pathologists could only agree in 5 cases and this would have resulted in three out of the seven patients who developed cancer not having a preceding diagnosis of LGD. As a result, although using three pathologists raises the predictive ability of LGD for future cancer development, it would result in a greater number of uncertain cases with more patients with cancer not having a prior diagnosis of LGD.

These problems with interobserver variation have contributed to the difficulties in comparing studies between centres in BO as well as the variation in reported rates of progression from HGD to adenocarcinoma. These difficulties have led to the recommendation that in routine clinical practice two histopathologists must agree on high grade dysplasia prior to oesophagectomy (Al Kasspoles et al, 2002).

## **1.5 Screening and Surveillance of Barrett's Oesophagus**

This remains, at best, controversial.

### ***1.5.1 Primary Screening***

The latest guidelines from the American College of Gastroenterology advise upper GI endoscopy in patients with longstanding GORD specifically for the detection of Barrett's (Sampliner, 2002). In the UK, however, the latest NICE guidelines suggested that only patients over the age of 55 years with persistent symptoms of reflux after acid inhibition should have an endoscopy (National Institute for Clinical Excellence, 2004). Furthermore, the recent BSG Guidelines suggested that at present 'endoscopic screening of patients with chronic heartburn to detect columnar lined oesophagus cannot be recommended', although, this recommendation is made on the basis 'of an absence of directly applicable studies' (Watson et al, 2005). These differences of opinion are not surprising as screening and subsequent surveillance of Barrett's oesophagus is unproven and there is no good evidence to suggest that primary screening either reduces mortality from oesophageal adenocarcinoma or is cost effective. Indeed this is the view of the AGA international workshop (Sharma et al, 2004). Two studies have tried to address this question. The first examined the cost effectiveness of screening GORD patients estimating the cost to be \$24,700 per life-year saved (Soni et al, 2000). The authors, however, rightly draw attention to the factors that might render this analysis invalid; small changes in the prevalence of Barrett's oesophagus (10% in the analysis), high grade dysplasia or adenocarcinoma (7%), any drop in the sensitivity or specificity of endoscopy (estimated as 90% each), and any rise above \$644 as the cost of the screening endoscopy and histology. Furthermore even a small drop in the quality of life associated with treatment (estimated overall 3.5% operative mortality with surgery and 50% early cancer cure rate) would significantly reduced the cost-effectiveness of screening.

The second study concluded that screening men over 50 years old with symptoms of GORD and then offering subsequent surveillance to patients diagnosed with dysplasia in Barrett's oesophagus would cost as little as \$10,440/life-year saved compared to no screening or surveillance. In patients without dysplasia however the cost of subsequent surveillance every five years rose to over \$50,000 per life year saved (Inadomi et al, 2003).



### ***1.5.2 Barrett's Surveillance***

The principle requirements for a successful surveillance program are:

1. The disease to be of significant burden to the population
2. Its incidence causes significant impact
3. That there is an acceptable form of surveillance
4. Which is accurate for detecting a high risk group
5. There is subsequently an acceptable form of treating the precancerous/cancer condition
6. The cost of surveillance is low
7. The risks of surveillance are significantly less than the benefits, and
8. There are adequate skills amongst professionals for delivery.

Surveillance might be cost effective but this is entirely dependent on the detection, prevalence and rate of progression from HGD to cancer. Sonnenberg et al in 2002, examined the feasibility of surveillance concluding that the incremental cost-effectiveness of surveillance endoscopy every six months is approximately \$17000/life-year saved. Again the authors highlight the dependence of this type of analysis on the incidence rate of oesophageal adenocarcinoma and the 5-year survival without surveillance chosen. The effect of a fall in the former and a rise in the latter would be to reduce cost effectiveness (Sonnenberg et al, 2002).

Surveillance suffers from a similar lack of randomised controlled data and although there are some studies reporting advantageous detection of early cancers and improved mortality the outcomes of these studies are mixed. The first of these studies showed that following oesophagectomy the cancer staging was significantly earlier in surveyed patients compared to non-surveyed patients. Also, in the surveillance group only 1/17 patients (6%) compared to 34/54 (63%) in the non-surveillance group had nodal involvement. This translated into an improved two year survival of 86% for the surveillance group compared to 43% of the non-surveillance group (Van Sandick et al, 1998).

Three other studies have shown that surveillance appears to allow the detection of cancers at an earlier stage with improved cure rates or survival rates as a consequence (Corley et al, 2002; Incarbone et al, 2002; Wright et al, 1996). Other studies, however, have not reported the detection of any curable cancers despite many years of surveillance (Conio et al, 2003).

It would seem that these studies are an optimistic assessment of the benefit and cost effectiveness of screening followed by surveillance in Barrett's. Garside et al in 2006, in an analysis commissioned by the Health Technology Assessment (HTA) in the UK, performed a more detailed analysis. The criteria used were conservative in the estimates of effectiveness with a 50% symptomatic cancer treatment rate, a 5% HGD regression rate to LGD and a 26% recurrence rate of cancer following surgery. The authors concluded however that the cost of screening followed by surveillance for all patients with Barrett's would cost around £45,000 per life year saved (Garside et al, 2006). The authors also estimated that treatment following surveillance did more harm than good due to high operative mortality and high recurrence rates. Inherently the uncertainty of all the factors entered into a cost effectiveness analysis make the interpretation of any study difficult but primary endoscopic screening for Barrett's with subsequent surveillance of all patients using dysplasia as the only risk-stratifier is almost certainly prohibitively expensive.

One of the cornerstones of any surveillance program remains the accurate detection of a high risk group and the diagnosis of Barrett's Oesophagus alone is clearly not accurate enough. These problems have led inevitably to doubts on both sides of the Atlantic as to whether Barrett's screening and/or surveillance is of any benefit at all (Garside et al, 2006; Sharma and Sidorenko, 2005) with the NHS recently commissioning a large multicentre randomised controlled trial to examine the question of surveillance in BO.

### ***1.5.3 What might make screening and surveillance viable?***

**Screening:** This requires targeting those in the population at highest risk of having BO and needs to be less expensive and more acceptable to the general public than endoscopy and biopsy. It is difficult not to be drawn to the conclusion that this method would have to be non-endoscopic, fast, risk-free, cheap and preferably automated.

**Surveillance:** More accurate risk stratification is crucial and at present is the single biggest stumbling block for surveillance. The identification of a low risk group that either require no further surveillance is of at least equal importance to the diagnosis of a *small* high risk group that require either intense surveillance or treatment.

**Treatment:** Oesophagectomy, with its adherent high risk of up to 7.5% mortality and up to 40% serious long-term morbidity, means that at present patients are faced with very difficult decisions were a surveillance program to place them at high risk. One must bear in mind that at this stage patients do not have oesophageal cancer. The spectre of surgery is a serious limitation at present

to surveillance and is the principle drive behind work into developing low risk yet effective minimally invasive ablative therapies.

## 1.6 Biomarkers in Barrett's Oesophagus

Histological determination of HGD is not the whole story in the development of adenocarcinoma of the oesophagus and for surveillance to become of real clinical value it needs to be able to deliver more information about a patient's risk of progression to cancer. The identification of biomarkers (such as aneuploidy, p53 inactivation (by 17p mutations), and abnormal expression of the proliferation markers such as Cyclin D1) raise the prospect of being able to more accurately define the group of patients most likely to develop cancer. While there have been an enormous number (over 200) of biomarkers found in Barrett's oesophagus less than 5% have been validated beyond phase 1 of development (the discovery phase) (Paulson and Reid, 2004). It is, therefore, useful to consider the steps necessary to deliver a biomarker to the clinical arena.

The Early Detection Research Network of the National Cancer Institute has defined five phases of biomarker development and these are summarised in table 1.3 (Pepe et al, 2001).

**Table 1.3: Stages of Biomarker Development**

Phase 1	Preclinical/Exploratory	The identification of potentially useful markers
Phase 2	Clinical Assay and Validation	Clinical Assay detects established disease
Phase 3	Retrospective Longitudinal	Biomarker detects disease early before it becomes clinical & a positive test is defined
Phase 4	Prospective Screening	Extent and characteristics of disease detected by the test and false positive rate identified
Phase 5	Cancer Control	Impact of screening on reducing the burden of disease on the population is quantified

Very few biomarkers have reached phase 4 validation in Barrett's oesophagus. These biomarkers include 17p loss of heterozygosity (LOH) and other p53 mutations, aneuploidy and tetraploidy (Paulson and Reid, 2004). These biomarkers require validation in multicentre prospective trials, but are possible targets for phase five development and subsequent cancer control in a Barrett's surveillance program.

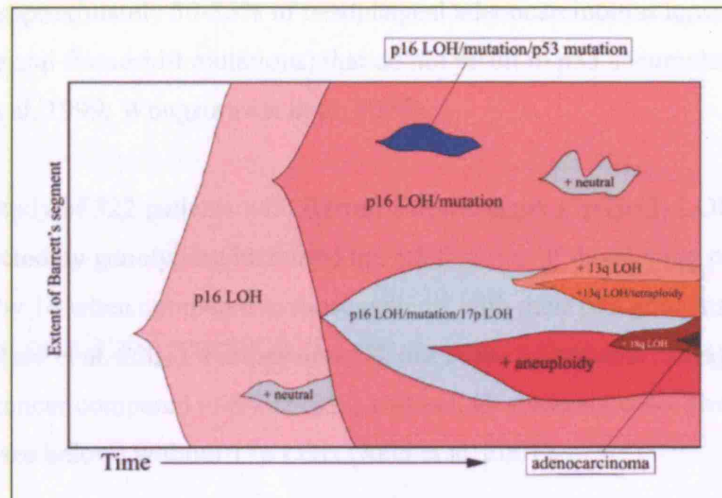
### **1.7 The Natural History of Genetic and Biomarker Abnormalities**

Clonal expansion in BO has been postulated as a concept by which a clone of cells has developed an evolutionary advantage over their neighbours. This advantage then allows this clone to spread through the Barrett's segment by the process of natural selection (Maley et al, 2004a; Maley and Reid, 2005). The two key factors suggested for this process were the ability of these premalignant clones to avoid apoptosis and their ability to ignore normal regulatory growth mechanisms.

It has been suggested that loss of p16 occurred early in the natural history of BO and was therefore not a good marker of future cancer risk because up to 85% of patients demonstrated this abnormality (Galipeau et al, 1999; Wong et al, 2001). One study, however, has suggested that p16 alterations might effect the length of the Barrett's segment (Wong et al, 2001). Irrespective of the exact timing that p16 alterations occur it would appear that p16 inactivation is a pre-requisite to future genetic lesions, particular p53 abnormalities. The Seattle group have also shown that p16 alterations spread throughout the Barrett's segment relatively quickly. This is an important concept because it exposes a greater number of cells to the risk of acquiring the next genetic abnormality required in the development of adenocarcinoma. See figure 1.2 below.

It has also been shown that 17p LOH affecting the TP53 gene locus occurs after the loss of p16 (Maley et al, 2004b) and that p53 abnormalities are probably crucial to the development of aneuploidy and tetraploidy. p53 inactivation disables the G1 checkpoint which has been suggested as the mechanism that permits uncontrolled access to division of karyotypically abnormal cells (Neshat et al, 1994; Reid et al, 2001). Furthermore, it was suggested that it was probably necessary for both p53 alleles to be lost in order for aneuploid and tetraploid clones to occur. Finally, aneuploidy and tetraploidy occur before the advent of adenocarcinoma (Barrett et al, 1999). In some, but not all cases, HGD was seen microscopically prior to the development of cancer. See figure 1.5 below.

**Figure 1.5: Proposed spread and development of genetic abnormalities in BO**



(Paulson and Reid, 2004)

## **1.8 Biomarkers that have reached Phase 4 of development in predicting future cancer risk**

### **1.8.1 p53 Inactivation (p17 LOH, TP53 mutation)**

The p53 cancer suppressor gene has been implicated as a biomarker for future cancer development. In a normal cell population if DNA damage occurred to the nucleus prior to division then p53 activation would cause cell cycle arrest and attempt DNA repair. If this fails then apoptosis occurred. Immunohistochemical detection of p53 showed a higher positively stained fraction with increasing levels of dysplasia. It has been noted that in non-dysplastic mucosa there is 0% positivity compared to 9-55% with dysplasia and 87% in adenocarcinoma. Furthermore, the p53 positively stained areas co-localised with the dysplastic areas of these biopsies (Younes et al, 1993). p53 immunostaining has also been shown to more accurately predict the future development of HGD or cancer than LGD. The presence of p53-positive biopsies were shown to have a higher positive predictive value of 0.56 compared to LGD at 0.2 for future cancer risk (Younes et al, 1997). This is supported by a second study showing similar results. Forty-eight patients with LGD at baseline were followed up for a mean of 42 months. During this period 5 progressed to HGD or cancer, 12 had persistent LGD and 31 had regressed to no dysplasia. p53 immunostaining was co-localised to areas of LGD in 3/5 (60%) that progressed, 3/12 (25%) that persisted and 4/31 (13%) of patients that regressed (Weston et al, 2001).

Immunostaining, however, may underestimate the true proportion of biopsies with p53 abnormalities as approximately 30-35% of oesophageal adenocarcinomas have p53 mutations (such as nonsense and frameshift mutations) that do not result in p53 accumulation (Hamelin et al, 1994; Prevo et al, 1999; Wongsurawat et al, 2006).

In a prospective study of 322 patients with Barrett's oesophagus 17p (p53) LOH (single gene inactivation) detected by genotyping increased the relative risk of developing oesophageal adenocarcinoma by 16 when compared to those patients with their p53 gene intact (p53 wild type/wild type) (Reid et al, 2001). Furthermore, 20 out of the 54 patients (38%) with baseline 17p LOH developed cancer compared to 6/202 (3%) without. Of these six cases two had aneuploid cell populations (see below) without 17p LOH (Reid et al, 2001).

Murray et al in 2006 performed a nested case control study within a cohort of 1670 Barrett's oesophagus patients. Amongst the cases 29 patients developed cancer and 6 HGD during a mean follow up of 2.3 years. The follow up in the control group was 3.9 years. If an initial biopsy showed TP53 immunostaining then the odds ratio of developing HGD or cancer was 12 compared to the controls. Only 32% of cases, however, were TP53 positive at baseline. If a patient stained positive for both TP53 and COX-2 their odds ratio was 27, although only 15% of cases had positive staining for both markers (Murray et al, 2006). In isolation, therefore, TP53 abnormalities may not be sensitive or specific enough to accurately identify patients who are at risk of developing cancer and therefore additional biomarkers are likely to be required.

### ***1.8.2 Aneuploidy and Tetraploidy***

Aneuploidy and tetraploidy are measures of altered total DNA copy number within the nucleus of the cell. Aneuploid and tetraploid populations of cells occur within biopsies taken from Barrett's oesophagus and one set of definitions from the Seattle group based on future cancer risk are:

Aneuploidy: A population of cells with abnormal DNA content, separated from the diploid peak, and representing more than 2.5% of cells.

Tetraploidy: A population of cells increased beyond the normal level present during stage G2 of the cell cycle (>6%) and with DNA content between 3.85 and 4.1N inclusive.

(Vaughan et al, 2005)

These definitions of aneuploidy and tetraploidy relate specifically to changes in the overall total quantity of DNA in the nuclei of a group of cells in the biopsy. In truth these are crude measures of alteration and make no judgment as to the normality of each chromosome or gene with that nucleus.

These definitions for flow cytometric abnormalities examine the proportion of abnormal nuclei not just presence of absence of any given genetic defect and have been shown to more accurately predict an individual's risk of developing cancer. In a landmark, phase 4 prospective biomarker study of 322 patients with Barrett's oesophagus, the Seattle group have shown that flow cytometry abnormalities demonstrating the presence of aneuploidy or tetraploidy in oesophageal biopsies is an independent risk factor for the development of cancer (Rabinovitch et al, 2001; Reid et al, 2000b). This study also showed that when HGD was combined with these flow abnormalities, a patient's risk of progression to cancer could be predicted far more accurately. If a patient had both HGD and aneuploidy/tetraploidy the risk of developing cancer within five years was 66%, compared to 42% with HGD alone and 28% with only aneuploidy/tetraploidy. No patient without HGD or flow abnormalities developed cancer within five years (Reid et al, 2000b). See table 1.4 below. This latter low risk group is of particular importance to surveillance programs because it accounts for approximately 87% of patients with BO who, if they could be identified, would only require a surveillance endoscopy once every five years at the most. Supporting evidence is provided by an independent group who performed a case control study of thirteen patients with 13 years follow-up. In the 5 cases that developed cancer all had aneuploidy at baseline compared to only 1/7 patients in the control group. Interestingly HGD was only detected in 2/5 patients before they developed cancer supporting the notion that HGD alone is an poor predictor of cancer risk (Teodori et al, 1998).

**Table 1.4: Cancer risk from Seattle Cohort dependent on HGD and Aneuploidy**

<b>Cancer risk</b>			<b>5yr cancer incidence</b>
<b>Sample n=322</b>	<b>No, indefinite or LGD (n=247) 5 year cancer incidence 3.6%</b>	<b>No Flow Abnormality (n=215)</b>	<b>0% (0/215)</b>
		<b>Aneuploidy/Tetraploidy (n=32)</b>	<b>28% (9/32)</b>
	<b>HGD (n=75) 5 year cancer incidence 59%</b>	<b>No Flow Abnormality (n=26)</b>	<b>42% (11/26)</b>
		<b>Aneuploidy/Tetraploidy (n=49)</b>	<b>66% (32/49)</b>

(Reid et al, 2000b)

Furthermore, the higher the abnormal chromosome number in an aneuploid peak, defined as the mode of that peak on flow histogram, the higher the risk of adenocarcinoma development. The most discriminatory cut off for five year cancer risk has been suggested as the presence of aneuploid peaks at  $>2.7N$  (Rabinovitch et al, 2001).

Finally, the extent of spread of genetic abnormalities through a patient's BO has also been shown to define risk. Spread was measured as the number of vertical centimetres a genetic abnormality was found in that patient's Barrett's. A multivariate analysis examined the development of cancer relative to the length of Barrett's with p16 inactivation, p53 LOH and aneuploidy/ tetraploidy. The latter two were significant while length of p16 abnormalities was not associated with future cancer risk. The hazard ratios in this analysis were:

p53 LOH:	1.27 x length of abnormality (cm), and
Aneuploidy/Tetraploidy:	1.31 x length of abnormality (cm).

(Maley et al, 2004a)

## **1.9 Chemotherapy in Barrett's Oesophagus**

### ***1.9.1 Proton Pump Inhibitors***

Proton Pump Inhibitors (PPI) use has been shown to reduce proliferation in BO (Ouatu-Lascar et al, 1999). This result has been confirmed in a separate study which suggested that it was a direct effect of acid suppression. This study of 51 patients on regular PPI measured ongoing acid exposure with pH monitoring and in the inadequately suppressed group proliferation was higher, measured using proliferating cell nuclear antigen.

Two studies have looked at PPI use and subsequent occurrence of dysplasia and adenocarcinoma. The first examined 236 patients with 1,170 patient years of follow up with 56 developing dysplasia. There was significantly less dysplasia development amongst those who used PPI as opposed to those with either no acid suppression or suppression with H2 receptor antagonists (El Serag et al, 2004). The second study surveyed a cohort of 350 patients followed-up for a median of 4.7 years. In those who delayed starting a PPI for more than 2 years the hazard ratio for developing HGD or adenocarcinoma was approximately 21 (Hillman et al, 2004).

Finally, the use of long term acid suppression with PPI may lead to partial regression of BO with squamous re-epithelisation. This re-epithelisation has also been shown to cause Barrett's



epithelium to become buried underneath regenerated squamous mucosa (Hornick et al, 2005). Proton pump inhibitors are now widely used in patients with Barrett's oesophagus.

### ***1.9.2 Aspirin and Cyclo-oxygenase 2 (COX2) inhibition***

Increased COX-2 expression was first reported in Barrett's Oesophagus when compared to gastric controls by Wilson et al in 1998 (Wilson et al, 1998). Subsequently, it has been shown that in 56 patients with Barrett's oesophagus epithelial expression of COX2, measured by immuno-histochemistry, was correlated with dysplasia (Morris et al, 2001).

Two further studies have linked high expression of COX2 in tumour samples with significantly higher rates of lymph node metastases and reduced survival (Buskens et al, 2002; Mobius et al, 2005).

Of greater importance perhaps is COX2 as a potential target for chemotherapy. Interest in specific COX2 inhibitors however has waned because of concerns over their cardiac safety profile but aspirin remains a viable alternative. One case control study of 293 patients with adenocarcinoma showed that current users of aspirin were at lower risk of developing oesophageal adenocarcinoma with an odds ratio of 0.37 (Farrow et al, 1998). Additional evidence comes from the Seattle group's prospective cohort of 350 patients with Barrett's oesophagus with median follow-up of 65.5 months. Thirty-seven patients developed cancer at 5 years follow up and data was prospectively collected about NSAID use. In a multivariate analysis, including all the known major risk factors, the incidence of adenocarcinoma and occurrence of aneuploidy and tetraploidy in the regular aspirin or NSAID users compared to non-users was significantly less with hazard ratios of 0.2 for cancer, 0.25 for aneuploidy and 0.44 for tetraploidy (Vaughan et al, 2005).

The use of both PPI and aspirin is now the focus of a major randomised controlled trial in the UK (AspECT) which aims to recruit 5000 patients over two years and follow them up for 8 years to assess all cause mortality, and oesophageal adenocarcinoma incidence between groups. The results of this study will increase our understanding of the true benefits to these therapies although one criticism already levelled at this study is that there is no true control group (no treatment with a PPI or aspirin). It is difficult however to get a true control group as many patients have symptoms of reflux or ongoing oesophagitis which require PPI therapy.

### **1.10 Summary**

In conclusion, the management of Barrett's Oesophagus has reached a difficult impasse with gastroenterologists in the western world unable to make an impact on the rising incidence of oesophageal cancer. Clinicians are unable to identify enough patients in the general population with BO for surveillance, unable to identify accurately those who will develop oesophageal adenocarcinoma and finally unable to offer them an effective, safe, acceptable treatment were they to be in this high risk group. Despondency at this situation is exacerbated by demands by either government or health insurers for delivering health care at ever lower cost. Improvements in both diagnostics and therapeutics either with biomarkers, in endoscopy or with medication would appear essential for a brighter outlook in this disease.

## Chapter 2

# Novel Technologies for the diagnosis of precancerous lesions in Barrett's Oesophagus

## **2 Novel Technologies for diagnosis of precancerous lesions in Barrett's Oesophagus**

### **2.1 The need for new technologies**

Oesophageal cancer rates are rising rapidly throughout the Western World (Lagergren 2005), presenting late when less than 25% of cancers are operable (De Vita et al. 2002; Fernandez and Meyers 2004; Keighley 2003). Clinicians have therefore focused on trying to detect patients at high risk of going on to develop adenocarcinoma by performing surveillance on those known to have Barrett's oesophagus. A patient's lifetime risk with Barrett's is probably only around 5% and therefore emphasis has been placed on the detection of dysplasia (Jankowski, Provenzale, and Moayyedi 2002; Shaheen 2001; Spechler 2000). Dysplasia although imprecise in its detection, diagnosis and prediction of future cancer risk is currently the only widely available method of risk stratification.

The patchy, occult and mosaic distribution of HGD means that the likelihood of detecting it is associated with the number of biopsies taken (Reid et al. 1992). Additionally, Van Sandick et al showed that sampling errors are more likely if insufficient biopsies are taken (Van Sandick et al. 1998). At present, even using the most rigorous biopsy protocols, only about 1% of a patients Barrett's oesophagus is examined histologically (Reid et al. 2000).

This patchy and occult distribution of HGD results in many problems for Gastroenterologists but most notably the anxiety of potentially 'missing' the premalignant tissue purely by sampling error. Furthermore, many studies have suggested that the occult malignancy rate is between 38% and 50% of patients with biopsy-proven HGD when their resection specimens are examined (Rice et al. 1993; Heitmiller, Redmond, and Hamilton 1996; Pera et al. 1992; Nigro et al. 1999; Altorki et al. 1990) although the long-term cancer incidence in patients with HGD from surveillance studies is 13-59% (Montgomery et al. 2001; Rabinovitch et al. 2001; Schnell et al. 2001; Spechler et al. 2001). Irrespective of the exact risk many clinicians believe it high enough to justify resecting the entire Barrett's segment if HGD is found with all of its significant attendant risks. These difficulties in detecting HGD and its imprecise cancer prediction not only reduce the effectiveness of any surveillance program but also provide clinicians with a dilemma as to how often a patient

should undergo surveillance endoscopies. One group has re-reported results from a more recent post oesophagectomy series for comparison with the harboured occult malignancy rate falling from 38% to 17% (5/30) in the period 1994-2001 (Tseng et al. 2003). This data might suggest that surveillance programs which are now advised by the American Society of Gastroenterology (Sampliner 2002) are detecting patients with HGD earlier in the metaplasia-dysplasia-carcinoma sequence. The current procedure of quadrant biopsies every 2 cm of BO is imprecise, time consuming both for endoscopies and histopathologists (Messmann et al. 1999) and is a relatively poor predictor of future cancer risk. The result of this is the NHS, amongst others, questioning the value of surveillance in Barrett's Oesophagus altogether (Garside et al. 2006).

If these problems were not daunting in themselves the diagnosis of dysplasia, the current gold standard, is subjective and needs an expert pathologist to interpret the biopsies (Fitzgerald and Triadafilopoulos 1998). It has been shown that even expert gastrointestinal pathologists only agree on HGD on approximately 85% occasions with them agreeing on LGD little more than 20% of the time (Reid et al. 1988; Lovat et al. 2006). The more pathologists, however, do agree on the histological findings then the greater the risk of future cancer development (Skacel et al. 2000). It is not practical to have multiple reporting of single slides, particularly in the UK, where cost and the lack of trained pathologists makes even double reporting of those slides demonstrating HGD difficult. Additionally many slides would be left in the uncertain category of indefinite for dysplasia when pathologists disagree.

In summary the principle problems that currently exist with surveillance are:

1. The 'random' nature of detecting patchy and occult dysplasia;
2. The large number of specimens required to reasonably sample a Barrett's segment;
3. The time required by both pathologist and endoscopists to perform these tasks;
4. The lack of agreement between pathologists in the diagnosis of dysplasia;
5. Dysplasia imprecisely defines future cancer risk;
6. Approximately 95% of patients will never develop cancer but still undergo surveillance;
7. The cost of running any surveillance program.

## **2.2 Novel Optical Technologies**

In an ideal world any novel diagnostic technique for use in Barrett's Oesophagus would provide the following advantages:

1. Allow a greater area of the mucosa to be examined,
2. Reduce the current length of time necessary to perform the endoscopy,
3. Remove any subjective interpretation of the results by pathologist or endoscopists,
4. Be compatible with existing endoscopes or require cheap additions,
5. Not require extended periods of additional specialist training,
6. Provide instant results within the endoscopy suite,
7. Be safe, and
8. More accurately detect high grade dysplasia (HGD) than standard systematic quadrantic biopsies and/or detect other biomarkers of future cancer risk.

This, at present, is an unrealistic specification list for any individual optical technique but forms a target for novel technologies to aspire to and many of these advantages would need to be fulfilled to demonstrate practicality in the clinical setting.

Novel optical technologies broadly fall into two categories: those that are point measurements similar to standard biopsy and those that provide visual information over an area resulting in imaging of a 'field'. These are not mutually exclusive groups but are useful for classification. Different field imaging techniques also assess greater and smaller areas but typically rely on expert training and subjective interpretation whereas point measurements may be very fast, reduce the need for expert interpretation but have the same 'sampling errors' as current histological methods.

**Table 2.1: Summary of Optical Techniques for the detection of neoplasia in Barrett's surveillance**

<b>Optical Technique</b>	<b>In Vivo/ Ex Vivo</b>	<b>Sensitivity for HGD/Ca</b>	<b>Specificity for HGD/Ca</b>	<b>Comments</b>	<b>References</b>
<b>Point measurements</b>					
Fluorescence:					
Autofluorescence	In vivo	74%-97%	39%-95%	Now largely used as an image device.	Panjehpour 1996, Pfefer 2003, Wang 2001
Drug Induced Fluorescence	In vivo	77%	71%	ALA the most promising drug	Brand 2002
Time gated Fluorescence	In vivo	76% for LGD	63% for LGD		Ortner 2003
ESS/Diffuse Reflectance/LSS	In vivo	84-100%	60%-100%	Varying size datasets. No prospective testing	Gurjar 2001, Wallace 2000, Georgakoudi 2003, Backman 2000, Lovat 2004 & 2006.
Raman	Ex vivo	88%	92%		Kendall 2003, Song 2000
	In vivo	88%	89%		Song 2005
Confocal	In vivo	93%	98%	Small study of 60 patients. Expensive Equipment	Kiesslich 2006, Kara 2007
<b>Combined 'Trimodal'</b>	In vivo	100%	100%	Small non prospective dataset	Georgakoudi 2001
<b>Image</b>					
Magnification and Chromoendoscopy	In vivo	93% 37%-87%	86% 46-91%	Indigo Carmine Methylene Blue (may result in DNA damage) LIFE	Kara 2005c, Sharma 2003. Egger 2003, Ormeci 2007 Gossner 2006 Olliver 2003
Autofluorescence	In vivo	21%-69% May increase detection x2	91% No data	AFI system (Olympus)	Egger 2003, Niepsuj 2003 Kara 2005c
Drug Induced Fluorescence	In vivo	60%-100%	27%-70%	Depending on route and dose of ALA	Mayinger 2001, Endlicher 2001, Stepinac 2003
Narrow Band Imaging	In vivo	93%	86%	Small study. Chromoendoscopy to direct biopsies	Kara 2006, Kara 2005a, Anagnostopoulos 2007
Optical Coherence Tomography	In vivo	68%	82%	Small study. Poor Interobserver agreement.	Poneros 2001, Iserberg 2005

## **2.3 Point Techniques**

### ***2.3.1 Fluorescence Spectroscopy (Point)***

#### **Autofluorescence**

Ultraviolet or banded white light (wavelengths shorter than green are screened out) is shone on to tissues, it is absorbed and then re-emitted (fluorescence) at a different wavelength. Different tissues are often excited by different wavelengths of light. The re-emitted light is at a longer wavelength than the incident light (for example, blue light is re-emitted as green).

Tissues can contain either light re-emitting particles (fluorescent chromophores) for example, NADH, elastin and collagen or light absorbing particles (non-fluorescent chromophores) for example, haemoglobin. Fluorescence is, therefore, dependent on the biochemical make up of the tissue. Dips and peaks in the spectral shape can occur as a result of absorption by non-fluorescent absorbing chromophores.

In clinical practice autofluorescence should theoretically demonstrate a difference between neoplastic areas and normal tissues as increases in endogenous chromophores produce a negative reaction in neoplasia.

Autofluorescence has been studied in the diagnosis of dysplasia and cancer in Barrett's oesophagus. Panjehpour et al first assessed the ability of autofluorescence to correctly classify Barrett's mucosa as either benign (no or low-grade dysplasia) or premalignant (HGD) in 308 biopsies taken from 36 patients of which 56 biopsies contained HGD. Autofluorescence excitation at 410nm correctly identified widespread HGD with a sensitivity of 97% and specificity of 39% from benign tissue (LGD and SIM) but this fell to a sensitivity of 28% for focal HGD (Panjehpour et al. 1996).

Subsequently two studies assessed the potential of autofluorescence for the detection of HGD in Barrett's oesophagus. One study used excitation wavelengths of 337nm and 400nm and diagnosed HGD in Barrett's oesophagus with sensitivities of 74% and specificities of 67% and 85% respectively (Pfefer et al. 2003). This suggested excitation at 400nm was slightly more effective



but still disappointing. Another study used spectroscopic ratios after excitation at different wavelengths. The most effective excitation ratio was 390nm/550nm which differentiated dysplasia and early carcinoma from normal oesophageal mucosa and Barrett's oesophagus with a sensitivity of 86% and specificity of 95% (Bourg-Heckly et al. 2000).

Wang et al also have presented data regarding the accuracy of autofluorescence in detecting HGD in BO. They detected this abnormality with 95% sensitivity and 80% specificity in 87 studied patients. The number of samples with HGD however was small at 14 out of 326 biopsies (Wang et al. 2001).

Time-resolved or time-gated fluorescence is able to identify some additional tissue features, for example, pH and oxygenation (Lakowicz et al. 1983). When light is shone at tissue if detection is delayed for approximately 20ns then the whole fluorescence spectrum has disappeared from normal tissues but the fluorescence decays more slowly in dysplasia. Promising levels of accuracy for dysplasia have been demonstrated in the detection of adenomatous polyps in the colon raising the possibility of its use in Barrett's oesophagus (Mycek, Schomacker, and Nishioka 1998). Time resolved autofluorescence at 550nm was used to provide data in one study but this was shown to be less effective than steady state fluorescence at both 337nm and 400nm for the detection of HGD in Barrett's oesophagus. Steady state autofluorescence excited with blue light at 400nm produced a sensitivity of 74% and specificity of 85% (Pfefer et al. 2003).

### **Drug-induced fluorescence (point)**

Drug-induced fluorescence (DIF) is dependent on the selective uptake of a drug in to the target tissue or disease and its principle advantage over autofluorescence is the generation of a comparatively strong signal. The subsequent procedure, however, requires careful timing for maximal efficacy. Drugs can be given orally, topically or intravenously. Topical treatments allow for administration during the procedure but this elongates procedure time. In some cases with topical administration of ALA two endoscopies would be required up to 3 hours apart; one for topical drug delivery and one for fluorescence measurement. This organisation is no mean feat on busy and potentially unpredictable NHS lists which would significantly hinder any widespread introduction into secondary surveillance programs.

The most widely studied drug is 5-aminolaevulinic acid (ALA). The principle reasons for the use of ALA are; it can be taken orally, in small doses there is little or no long-term photosensitivity, it has preferential mucosal uptake especially in HGD and carcinoma and, in these doses, appears safe.

One study examined the detection rate of HGD using DIF following oral administration of 10 mg/kg ALA 3 hours prior to endoscopy. The fluorescence intensity at 635 nm was measured and high-grade dysplasia was distinguished from non-dysplastic tissue with a sensitivity of 77% and a specificity of 71%. Furthermore, if a fluorescence intensity ratio of 635 nm/480 nm was calculated, nodular HGD could be detected with a sensitivity of 100% and a specificity of 100% (Brand et al. 2002). All 5 patients with nodular HGD, however, were also correctly identified with normal white light endoscopy and biopsy and when endoscopy plus random biopsy were combined 10/13 patients were correctly diagnosed with HGD, the same as fluorescence endoscopy. The sensitivity for the detection of flat, non-nodular HGD was 62% (5/8 patients) with random biopsy and fluorescence spectroscopy.

### **Time-gated drug induced fluorescence**

An intensity ratio of fluorescence (delayed 20ns) compared to immediate fluorescence has also been examined in 53 patients for the detection of low grade dysplasia. The oesophageal mucosa was sprayed with ALA 60-120 minutes prior to intensity ratio measurement and LGD was detected with a sensitivity of 76% and a specificity of 63%. Dysplasia was detected in 2.8 times more patients with time-gated fluorescence compared to normal endoscopy (Ortner et al. 2003). As previously discussed however the interpretation of LGD is difficult because of significant inter-observer variability between pathologists and the lack of a clear and significant progression rate from LGD to oesophageal carcinoma.

This study, therefore, did not provide clear evidence for the use of time-gated fluorescence. It suggested that it is not significantly better than other fluorescence based techniques and made no assessment of high risk lesions which would be of more clinical relevance regarding future cancer risk.

Overall autofluorescence and DIF as point measurements provided disappointing results and these have consequently been replaced by technological improvement in the power of the light sources to allow these techniques to be deployed for field imaging.

### ***2.3.2 Elastic and Light Scattering Spectroscopy***

Elastic Scattering Spectroscopy (ESS) is based on white light reflectance and the absorption of light by the tissue. Incident photons that are not absorbed by tissue are back-scattered at the same wavelength from the tissue (ie elastic scattering). These photons are then collected by a second fibre in the same optical probe. This back scattered light is subjected to spectral analysis providing structural information about the interrogated tissue potentially without need for tissue removal.

ESS, using a small separation between the two fibre tips, is more sensitive to scattering events than to absorption. As this fibre centre to centre separation increases then the technique is more sensitive to absorption becoming almost completely insensitive to small changes in the scattering coefficient of tissue when the centre to centre separation is about 1.7mm (Mourant 1997). The majority of scattered incident photons are scattered in an elastic way and, therefore, the intensity of signal is large relative to other optical techniques, such as Raman Spectroscopy. Light scattering spectroscopy (LSS) is a variation of ESS which examines only singly scattered or directly back-scattered light. This technique uses the principle of light polarity which is changed by multiple scattering events. By only collecting back-scattered light of the same polarity as that of the incident light only single or minimally scattered photons are recorded (Gurjar 2001).

ESS has developed into a real-time, in vivo, technique which detects changes in the physical properties of cells. Mourant et al have showed that high angle scattering of light >40 degrees is principally due to the nucleus and >110 degrees is correlated with DNA chromatin content (Mourant et al. 2000; Mourant et al. 1998). An independent group has also shown that the scattering signature collected using LSS was sensitive to nuclear enlargement, crowding and hyperchromicity (Gurjar et al. 2001; Georgakoudi and Van Dam 2003; Wallace et al. 2000). Furthermore, it would seem that singly scattered light (LSS) also identified increases in nuclear size, density and chromatin content (Backman et al. 2000). These are some of the main features that pathologists use for diagnosing dysplasia.

In a small series LSS spectra were taken from seventy-six sites in 13 patients. Four sites from 4 different patients showed high-grade dysplasia (HGD), 8 sites from 5 different patients showed low-grade dysplasia (LGD), 12 sites were indefinite for dysplasia, and 52 sites showed nondysplastic Barrett's mucosa. LSS was shown to detect HGD in Barrett's oesophagus with a sensitivity of 90% and specificity of 90% in these samples (Wallace et al. 2000). LSS has also been subsequently used in 16 patients with 40 samples collected of which 7 displayed HGD. LSS had a sensitivity of 100% and specificity of 91%. In this study both autofluorescence and diffuse reflectance spectroscopy (essentially a different name for ESS which measures multiple elastic scattering of light) were also used as a trimodal spectroscopic point assessment. Individually autofluorescence had a sensitivity of 100% and specificity of 97% and ESS had a sensitivity of 86% and specificity of 100%. When these individual techniques are combined then a sensitivity and specificity of 100% are obtained (Georgakoudi et al. 2001).

This study while encouraging only recruited 7 sites with HGD and with multiple (at least 7) output variables collected (3 measures with fluorescence, the scattering coefficient slope and intercept with diffuse reflectance and estimates of the nuclear size and cellular crowding with LSS). This raised the possibility of the introducing a statistical error called 'overfitting' and is a problem of any advanced statistical methods employed to analyse high dimension data (see section 6.5.1).

In a preliminary study from this unit ESS spectra were collected from 41 patients and 71 sites. After a block validation statistical analysis ('bootstrap' analysis) this initial dataset suggested a sensitivity of 75% and specificity of 80% for the detection of HGD in BO (Lovat and Bown 2004).

Four dimension elastic light scattering fingerprinting (4D-ELF) is an advanced elastic scattering method which in preliminary animal and human studies has been shown to be sensitive to changes in the mucosal and submucosal blood flow with depth resolution (Roy et al. 2004; Wali et al. 2005). This technique works by shining polarised light into the tissue and by only collecting backscattered light at very high angles of scatter (re-entering the spectrometer at an angle of 0-7 degrees). Additionally, by polarisation gating of the light, information from the mucosa and submucosa can be distinguished (Roy et al. 2006). This group has demonstrated that 4D-ELF can accurately predict increased blood supply in the mucosa and submucosa in animal studies with a sensitivity of 94% and a specificity of 96% and that this change in blood supply predated the

occurrence of aberrant crypt foci. In a small preliminary series of 37 patients this study showed a three fold increase in mucosal blood flow in the transverse colon of patients who had large or severely dysplastic adenomas *elsewhere* in their colon (Roy et al. 2006). This was a very exciting result but caution must be applied with such small numbers of patients in the study. Additionally, the measurements were collected immediately ex vivo on biopsy specimens from the transverse colon during colonoscopy. In reality measurements would need to be collected for a screening test from a rectal biopsy. This study does, however, provide proof of concept for 4D ELF diagnosing a field effect in the colon that might be associated with future cancer risk.

One of the principle advantages of ESS is that measurements are collected in less than 200 milliseconds and as a result multiple ESS spectra can be collected much faster than tissue for histology especially during surveillance procedures when multiple biopsies need to be taken. Other potential advantages include: the ESS equipment is easy to use and is already compatible with normal endoscopes, the optical probes and spectral analysis equipment are not expensive or difficult to manufacture and because the analysis is based on computer algorithms there should be no inter-observer variability.

The two most significant limitations of ESS at present are the lack of prospective studies of the generated algorithms and that, like normal histological biopsy, this technique only examines the mucosa directly underneath the optical probe and does not 'sample' a larger area of the oesophagus with one reading making it prone to sampling error similar to standard biopsy protocols (Cameron and Carpenter 1997). The real discriminatory value of these elastic scattering techniques will only truly be apparent in prospective testing. A preliminary report of this prospective test using this trimodal technique was presented in abstract form in 2005 and the sensitivity and specificity at that stage had dropped to 90% (Wallace et al. 2005). Finally, with regard to trimodal spectroscopy and 4D ELF the equipment is expensive and it is not currently possible to perform 4D ELF measurements through the endoscope.

### **2.3.3 Raman Spectroscopy**

Raman spectroscopy measures the inelastic scattering of light and was first demonstrated in 1928. Inelastic scattering is a process by which an incident photon interacts with the electron cloud surrounding an atom resulting in a small amount of energy transfer causing excitation of the molecule to a higher vibrational state. As a result of this interaction and subsequent small energy

transfer, a photon of longer wavelength is re-emitted when the vibrational state reverts, a phenomenon known as Raman Scattering. Energy is proportional to frequency and, therefore, the light is shifted to a lower frequency as only a proportion of the incident photons energy is transferred in this way (ie emitted at a longer wavelength). The emitted signal is very weak because only a very small proportion of light interacting with tissue results in the inelastic scattering (compared with elastic scattering and autofluorescence which are up to a million times and 1000x stronger respectively). This results in two problems; the first is that Raman spectra in the visible wavelengths are obscured by the much larger fluorescence signals and the second is that readings take a comparatively long time to collect. The former problem can be circumvented by collecting spectra in the infrared or ultraviolet ranges where fluorescence does not occur or in the near-infrared spectrum where fluorescence generates a very weak signal. The second remains a significant problem for the translation of this method into clinical practice. The principle difference between Raman scattering and fluorescence is that Raman is not dependent on the absorption of light.

It is technically possible for a photon of shorter wavelength to be re-emitted although this is much less likely as the molecule must already be in an excited vibrational state. In this reaction kinetic energy from the molecule is passed to the emergent photon.

Raman spectral characteristics are drawn from the atomic nuclei and chemical bonds and, therefore, it is an analysis of the biochemical make up of tissues including amino acids, nucleotides and lipid distribution. For example, ultraviolet resonance Raman spectroscopy has been shown in colonic tissues to identify lower amino acid/nucleotide ratios or a lower level adenyl (A) signal in 10/11 neoplastic samples when compared to normal mucosa (Boustany et al. 1999). Additionally, Raman spectral differences which equate to decreased glycogen, increased nucleic acids, decreased carotenoids and changed protein conformation have been shown to occur in the development of malignancy (Kendall et al. 2003).

At present most studies have not collected Raman spectra in vivo because readings take a comparatively long time to collect. It has been possible however to collect spectra from the human gastrointestinal tract during routine clinical endoscopy with a good signal-to-noise ratio obtained after 5 seconds (Shim et al. 2000). This is considerably faster than many other Raman systems but still remains a significant problem, especially in the oesophagus, where it is difficult to maintain a stable position for this duration of time.

In ex vivo samples taken from 44 patients with Barrett's oesophagus it has been shown that Raman spectroscopy can predict HGD with a sensitivity of 88% and specificity of 92% when compared to consensus histopathology findings (3 pathologists). For adenocarcinoma Raman spectroscopy demonstrated a sensitivity of 73% and specificity of 99% (Kendall et al. 2003). A second Raman analysis of, ex vivo, biopsies from Barrett's oesophagus correctly identified dysplasia with a sensitivity of 75% and specificity of 92% when compared to histology (Song et al. 2000).

There has been one recent study of in vivo measurements using Raman spectroscopy in Barrett's oesophagus on 65 patients. 26 out of 192 biopsy sites showed HGD or early cancer and Raman correctly identified these with a sensitivity 88% and specificity of 89% (Song et al. 2005).

It would appear, therefore, that the technical barriers to in vivo measurements with Raman are being bridged and again although the datasets are small this data would appear to demonstrate comparable accuracy to many other optical techniques. Raman Spectroscopy might provide a useful adjunct to other optical systems for multi-modality measurements because Raman provides information about the biochemical make up rather the physical properties of tissue. One further serious limitation at present is the cost of a Raman system.

#### ***2.3.4 Confocal microendoscopy***

Confocal microendoscopy is a high-magnification system providing the endoscopist with a cross-sectional image of the gastrointestinal epithelium during endoscopy on a similar scale to histology (Evans and Nishioka 2005). It is now possible to generate images with a magnification of over 1000x during endoscopy (Inoue 2005). No studies have examined the accuracy of this technique in Barrett's oesophagus but some authors suggest that, for the diagnosis of dysplasia in Barrett's, dye staining may be necessary with either fluorescein sodium (intravenous) or cresyl violet (topical). Confocal microendoscopy has been used in the colon with excellent accuracy. Kiesslich et al studied 60 patients prospectively having intravenous fluorescein sodium yielding high quality images. Detailed analysis of cellular structures demonstrated that dysplasia was associated with changes in the cellular and vascular patterns. Neoplastic locations were identified with a sensitivity of 93% and specificity of 93% and the interobserver agreement in this study was good at 0.84 (Kiesslich et al. 2006).

One study has combined confocal microendoscopy with autofluorescence and examined Barrett's mucosa ex vivo excitation occurred at 458 nm and green and red light emissions were collected at 505-550 nm for green and >560 nm for red. Unfortunately there were no specific changes associated with dysplasia such as intensity of either light emissions or their ratios. These findings led the authors to conclude that autofluorescence changes associated with dysplasia are not related to epithelial fluorophores but are more likely to be related to changes, such as mucosal thickening and increased microvasculature, in the collagen-rich submucosa (Kara et al. 2007).

The main drawbacks likely to occur with confocal microendoscopy are that the equipment is expensive, the images require expert interpretation and there is likely to be significant inter-observer variability outside expert units. Furthermore, it may be necessary to prepare an area with dye staining or by previously having given intravenous contrast.

## **2.4 Field Imaging**

Intuitively, a novel optical technique that is able to provide information about an area of Barrett's oesophagus would provide significant advantages. Field Imaging techniques, therefore, would appear to fulfil at least the first criteria detailed above but their ability to improve the sensitivity for the detection of HGD or indeed increase the numbers of patients diagnosed with HGD or intramucosal cancers over standard surveillance endoscopy, needs to be proven.

### ***2.4.1 Magnification and Chromoendoscopy***

Magnification, zoom or high resolution endoscopy has improved significantly in recent years with the delivery of improved technology. The most significant improvement has been in the advance of endoscopic optical (as opposed to digital) magnification. The number of pixels per field has also increased and the fields of view have become larger and wider. Digital magnification is now consequently becoming of some use as an add on to optical magnification but remains limited as this reduces the number of pixels in the field and is, therefore, offset against image clarity.

Chromoendoscopy is the addition of a substance to the surface of the gastrointestinal tract to enhance visualisation of the mucosa. When magnification is combined with chromoendoscopy, particularly in



the oesophagus, the procedure becomes more accurate as a diagnostic tool. At present, one significant advantage of chromoendoscopy over many other endoscopic optical techniques is its potential to allow a greater area of mucosa to be examined at one time. This field imaging increases the percentage of mucosa inspected during the procedure.

Chromoendoscopy uses two main types of staining: 'Absorptive' and 'Contrast' Staining.

**Absorptive Staining:** This is the addition of a substance that reacts with elements of the mucosa in order to distinguish it from different adjacent areas of mucosa for the diagnosis of inflammation, intestinal metaplasia, dysplasia and carcinoma. Examples of absorptive stains include:

- a) Toluidine Blue,
- b) Methylene Blue,
- c) Acetic Acid, and
- d) Cresyl Violet

a) **Toluidine Blue** selectively stains cell nuclei and cells with a high nuclear:cytoplasmic ratio stain more intensely (ie those with active or increased DNA synthesis). There has only been one clinical study examining the use of toluidine blue in Barrett's oesophagus and this showed an increased yield and enhanced detection of SIM in endoscopic biopsies targeted by chromoendoscopy (Chobanian et al. 1987). No assessment was made with regard to HGD or early carcinoma.

b) **Methylene blue** is taken up by the cytosol in intestinal metaplasia staining this type of mucosa dark blue. It does not stain non-absorptive mucosa (such as squamous or gastric mucosa). Dysplasia and carcinoma have lost some of their absorptive ability comparatively and, therefore, stain lighter blue.

Methylene blue is the most studied chromoendoscopy agent in Barrett's oesophagus. Canto et al, in 1996, first showed that methylene blue stained the specialised intestinal metaplasia (SIM) of Barrett's oesophagus with an accuracy of 95% in a study of 26 patients (Canto et al. 1996). In this study the authors also noted that dysplastic areas showed no or weaker staining with increased heterogeneity. This early study appeared to support further examination of chromoendoscopy for the targeting of biopsies with a view to reducing the number of biopsies required and possibly

enhancing the detection rates of high risk lesions (such as HGD or intramucosal carcinoma) in Barrett's Oesophagus.

Further studies, however, were disappointing. Several have assessed the ability of methylene blue staining to direct biopsies (MBDB) for dysplasia. Firstly, Canto et al in 2001 used MBDB which led to a higher proportion of biopsies containing dysplasia or cancer (44%) than random biopsies (24%) but most of these additional diagnoses were LGD. Furthermore 2 out of 43 patients had a 'missed' diagnosis of HGD (1 patient) or intramucosal adenocarcinoma (1 patient). In total HGD or adenocarcinoma was found on 3% of MBDB as opposed to 0.5% of random biopsies. This was not an overwhelmingly significant advantage when offset against the additional time and effort involved with dye staining at around 5-10 minutes per endoscopy (Canto et al. 2001).

There are two randomised crossover trials of MBDB versus conventional biopsies in the diagnosis of dysplasia in BO and these have shown similar detection rates for dysplasia with both techniques. One of these studies established a diagnosis of dysplasia in 12% of patients with MBDB and 10% of patients with conventional random biopsies (Ragunath et al. 2003). The second study showed that the frequencies for dysplasia were 20% from MBDB and 18% from conventional biopsy specimens however almost all of these patients had indefinite or low-grade dysplasia (Wo et al. 2001). LGD and indefinite for dysplasia are much more difficult diagnostic entities than HGD resulting in poor pathologist agreement and even less accurate prediction of future cancer risk. These studies therefore showed no significant benefit to staining with methylene blue.

A prospective study compared the potential of MBDB to autofluorescence and random biopsies. This study of 35 patients showed that the sensitivity and specificity for the detection of HGD or cancer were 37% and 91% respectively for MBDB and 21% and 91% for autofluorescence. Furthermore, standard endoscopy with quadrantic biopsy revealed five cancer or HGD areas and 76 LGD areas not detected by the other two methods. MBDB and autofluorescence detected only an additional one HGD area (in a patient correctly diagnosed with cancer elsewhere) and seven LGD areas compare to random biopsy (Egger et al. 2003).

Recently Gossner et al compared methylene blue directed biopsies with subsequent quadrantic biopsies in 86 patients with previously diagnosed HGD. In the MBDB group they re-identified more lesions than with quadrantic biopsies every 2cm. This is indeed encouraging but there was a

heavy bias towards early cancer (n=67) compared to HGD (n=17). Furthermore, this population all had pre-existing neoplasia and ultimately MBDB needs to be tested for its predictive ability in a surveillance group (Gossner et al. 2006). Another group has also compared MBDB (6 per patient) to a fixed set of 6 untargeted biopsies from BO in 109 patients. Despite not using a standard quadrantic biopsy protocol every 2cm this group failed to show a significant advantage for the detection of inflammation or oesophageal cancer in BO with MBDB (Ormezi et al. 2007).

There is no consistent evidence, therefore, to demonstrate a significant benefit of MBDB over quadrantic biopsies in the diagnosed of HGD or IMCA in a surveillance population. Furthermore, it has been suggested that methylene blue might induce oxidative damage to DNA in Barrett's mucosa, an effect of photoactivation dependent on the presence of both methylene blue and endoscopic white light. If proven this would be extremely concerning as Barrett's oesophagus is already considered to be a pre-malignant, genetically unstable condition which subsequently could progress to cancer at an increased rate with repeated methylene blue staining (Olliver et al. 2003). Interest has, therefore, largely moved on to newer dyes used in combination with magnification endoscopy.

c) **Enhanced magnification endoscopy with and without the addition of acetic acid** has been shown to increase the diagnostic yield of SIM in Barrett's oesophagus. Additionally, four distinct mucosal surface patterns have been described: round pits, reticular, villous and ridged. SIM in Barrett's oesophagus was detected according to the endoscopic patterns seen, as follows: Round Pits: 0%, Reticular: 11%, Villous: 87%, and Ridged: 100%. Standard endoscopy without biopsy would have missed 61/63 of these cases (Guelrud et al. 2001). Two recent studies, however, have combined acetic acid staining and magnification endoscopy. The first reports a sensitivity of 96% and specificity of 42% for the detection of SIM in 28 patients but among the six patients with HGD distinctive mucosal patterns were noted in only three (Reaud, Croue, and Boyer 2006). The second study of 64 patients also had small numbers of high risk patients (one with HGD and 3 with adenocarcinoma) making interpretation of this techniques ability to detect high risk patients difficult. Again inter-observer agreement in assessing pit patterns was at best only moderate with a kappa score of 0.57 (Fortun et al. 2006).

d) **A new dye, Cresyl violet**, has recently been used as a chromoendoscopy agent in conjunction with magnification endoscopy in a study of 1030 patients suspected of having Barrett's oesophagus. Of the 816 demonstrated to have Barrett's only 25 biopsies showed dysplasia of which 9 were carcinomas and 8 had HGD. The authors also propose a new simpler classification

of the pits as either 'open or closed' with an 'open' pattern representing both Barrett's and dysplasia. There remain many questions unanswered, however, such as the length of time taken to perform an endoscopy using Cresyl violet staining, how this test compared to quadrantic biopsies, the inter-observer agreement (a consensus of three expert endoscopists were used to interpret the images the this study) and a comparison of the effect of the two different regimens of dye staining used (0.05% Cresyl violet and 0.03% Cresyl violet plus 3.0% acetic acid) (Yuki et al. 2006).

This study led to a commentary entitled 'Is Cresyl violet the magic bullet?' with the authors providing an unreserved 'No'. This however was a preliminary study presented as such and perhaps a more balanced answer would have been 'No, not yet' (Kiesslich and Neurath 2006a).

***Contrast staining with Indigo Carmine:*** Contrast agents remain within the small structures on the mucosal surface. This staining relies on colour change, disruption of the vascular network and disruption of the innominate groove pattern as diagnostic criteria.

Magnification endoscopy after spraying with indigo carmine has demonstrated three types of mucosal patterns within columnar mucosa: ridged/villous pattern, circular pattern, and irregular/distorted pattern. The yield of SIM on targeted biopsies according to the mucosal patterns were; 97% with ridged/villous and 17% with a circular pattern. Six patients had an irregular/distorted pattern and all of these patients had HGD on biopsy. Eighteen patients had LGD on targeted biopsies all of whom had the ridged/villous pattern (Sharma et al. 2003b). This study is encouraging for the targeting of biopsies for SIM and HGD in Barrett's oesophagus but pattern recognition requires careful endoscopic skill, there is no mention of inter-observer variability, only a small number of patients in this study had HGD and therefore sensitivities and specificities were not estimated.

A randomised crossover study has compared high resolution endoscopy with indigo carmine staining to narrow band imaging. Twenty eight patients underwent each of the two techniques 6-8 weeks apart performed by a different endoscopist blinded to the previous results. Fourteen out of 28 patients were found to have HGD or early cancer. The sensitivity of both techniques were comparable with Indigo carmine chromoendoscopy displaying a sensitivity of 93% compared to a sensitivity of 86% with narrow band imaging (Kara et al. 2005c).

The current limitations of magnification and chromoendoscopy are:

- a) The procedure is more time-consuming and less well tolerated than unassisted endoscopy (Dave, Shousha, and Westaby 2001).
- b) It is expensive and sophisticated equipment is required for magnification although most dyes are inexpensive.
- c) Experience is required both with the staining agents (eg in recognition of lesions) and with the magnification endoscope as it must be held steady during magnification. Findings are also subjective and consequently accurate diagnosis is operator dependent. At the gastro-oesophageal junction one study examined this inter-observer variability. Strict evaluation criteria were established and classification was carried out according to the pit-pattern structure, methylene blue positivity, and the presence of villous structures. A high level of inter-observer variability was seen among the four experienced examiners (all kappa values < 0.4). SIM was histologically detectable in 61% of the patients and the accuracy of all of the examiners for predicting SIM by magnification endoscopy was around 50%, with no differences observed before and after instillation of acetic acid or methylene blue (Meining et al. 2004).
- d) The evidence to date would not suggest that the use of chromoendoscopy increases the diagnostic yield of HGD in Barrett's oesophagus nor has it been consistently shown to reduce significantly the number of biopsies required to detect HGD.
- e) The final and perhaps most significant limitations of magnification and chromoendoscopy in Barrett's oesophagus are the confusing number of staining techniques, the many different staining agents and the complex, inconsistently described staining patterns of the mucosa (Kiesslich and Neurath 2006b).

In summary, six of the eight benefits, described above, that a novel optical technology could deliver to a clinician are at least unproven if not unfulfilled. These reasons have prevented the widespread uptake of chromoendoscopy in district general hospitals, particularly for BO and its implementation into surveillance programs.

### **2.4.2 Fluorescence Imaging**

#### **Autofluorescence**

The development of fluorescence imaging has been largely dependent on overcoming the technical limitations of the endoscope, a field which is moving forward with breathtaking acceleration. Haringsma et al in 2001 described the feasibility for detecting dysplasia and early cancer unapparent to white light endoscopy by studying a field of Barrett's mucosa. This technique was termed Light Induced Fluorescence Endoscopy (LIFE) and autofluorescence was demonstrated following excitation using a blue light source and detection of green (490-560nm) and red (>630nm) fluorescence (Haringsma et al. 2001). More recently autofluorescence imaging (AFI) has combined autofluorescence with green and red reflectance (540-560nm and 600-620nm respectively) significantly enhancing the quality of images (Kara et al. 2005b).

There have been three studies published that have attempted to quantify the ability of LIFE to detect HGD or early cancers in Barrett's oesophagus. The first by Egger, detailed above under chromoendoscopy, was a prospective study comparing the potential of autofluorescence to methylene blue directed biopsies and random biopsies under white light endoscopy. This study of 35 patients showed that the sensitivity and specificity for the detection of HGD or cancer was worst for autofluorescence at 21% and 91% respectively. Furthermore, standard endoscopy with quadrantic biopsy revealed five neoplastic areas not detected by the other two methods (Egger et al. 2003).

The efficacy of autofluorescence endoscopy for the detection of HGD in short segment Barrett's oesophagus was assessed using excitation with blue light (425-455 nm) in a study of 34 patients. Autofluorescence detected HGD in 7 patients, 6 more than were identified with white light endoscopy and random biopsy alone. This result was interesting but suggested that the miss rate for HGD with random biopsies is 86% which has not been reproduced in any other Barrett's surveillance or optical imaging study. This group (Niepsuj et al. 2003) suggests that fluorescence guided biopsies were obtained prior to standard biopsies which could have influenced negatively the accuracy of random biopsies with the latter therefore being collected from different areas. The more likely explanation however surrounds that fact that on average only 4 random biopsies were collected per patient (136 biopsies from 34 patients and 109 targeted biopsies were collected using LIFE).

A third study has examined the prospect of enhanced detection of HGD or early cancers in Barrett's oesophagus. In a blinded randomised crossover design 50 patients underwent standard endoscopy and quadrantic 2cm biopsy or LIFE with targeted biopsies. Overall 15 patients were detected with high risk lesions, either HGD or early cancer. The overall sensitivity for standard endoscopy was 85% for high risk patients compared to 69% for LIFE ( $p=0.69$ ) (Kara et al. 2005c).

The same group has also tested the more recent AFI system (Olympus Optical Co, Tokyo, Japan) which combines AFI and reflectance in three settings: regular surveillance, evaluation of newly diagnosed HGD or early cancer and following ablative therapy. The images obtained appeared to the authors far more focused and clearer than with the LIFE system. 22 out of 60 patients were found to have HGD or early cancer of which 14 were detected by white light endoscopy and quadrantic biopsies every 2cm and a further additional 8 cases after the use of AFI suggesting approximately a 50% 'miss rate' for high risk lesions with quadrantic biopsies alone. A sampling error with the Seattle Biopsy Protocol is well documented but estimates vary as to its size between 20% and 50% (Kara et al. 2005c; Reid et al. 2000). This increased rate of detection if proven in independent studies is really very encouraging and may truly reflect the enhanced detection rates with this technique and also show the true scale of current sampling error with quadrantic biopsies taken every two cm. One caveat regarding this study's results is that, despite reporting being performed in a blinded way, the number of patients diagnosed with HGD was high at 33% with the histology reported by a single pathologist (Kara et al. 2005a).

### **Drug Induced Fluorescence Imaging:**

5-aminolevulinic acid (ALA) has also been used in imaging a field in an attempt to improve detection rates of precancerous lesions in Barrett's oesophagus. ALA-induced fluorescence has been studied in the detection of HGD and intraepithelial carcinoma by both oral and topical drug administration. Mayinger et al in 2001, compared drug induced fluorescence (DIF) with white-light endoscopy 6-7 hours post oral administration of 15mg/kg of ALA in 9 patients. Red fluorescence was exhibited by 85% of the biopsy sites with premalignant or malignant histopathology whereas only 25% of these biopsy sites were detected with white-light endoscopy alone (Mayinger et al. 2001).

A comparative study of 47 patients examining endoscopic DIF detection of dysplastic lesions in Barrett's oesophagus depending on the route and dose of ALA administered has also been performed. ALA was either given orally in doses of 5, 10, 20 or 30 mg/kg or topically 500mg or 1000 mg by spraying the mucosa via a catheter. Endoscopic fluorescence detection was performed 4-6 hours after oral and 1-2 hours after local administration. Sensitivity for detection of dysplastic lesions ranged from 60% after topical spraying with 500 mg to 100% after oral 5-ALA at 20mg/kg and 30 mg/kg. However, specificity was best for local topical ALA spraying at 70% while oral administration showed values falling to 51% and 27% respectively. The most suitable dosage for clinical application in Barrett's appeared to be 20mg/kg orally (sensitivity 100%, specificity 51%) leading significantly towards sensitivity in order to prevent 'missed' cases. This excellent sensitivity came at the expense of sending more biopsies to the histopathologists. Three patients, two with early cancers and one with dysplasia, were detected by DIF that were not detected during routine endoscopy (Endlicher et al. 2001).

A second comparative study of 28 patients compared white light endoscopy with DIF with ALA. In this study there was no significant advantage with DIF in the detection of HGD and neoplasia in Barrett's oesophagus. 5-aminolevulinic acid at 20 mg/kg was given orally 5 hours prior to endoscopy and both procedures correctly identified all patients with HGD or adenocarcinoma. DIF endoscopy however did result in significantly fewer biopsies taken (81 verses 531 biopsies) when compared to four-quadrant random biopsy every 2cm (Stepinac et al. 2003).

These studies would, therefore, suggest that the primary use for both autofluorescence and drug induced fluorescence, at present in Barrett's oesophagus, is in the targeting of biopsies to reduce the number needed to be taken but modern autofluorescence imaging may increase the detection rate of HGD by reducing sampling error. There are of course drawbacks. Both AFI and DIF techniques, especially the latter, are likely to increase the length of the endoscopy and in the case of DIF require additional prior drug administration. Both require expensive equipment, require addition specialist training and are prone to inter-observer variability although no comparative data exist to quantify the effect of this variability.

#### ***2.4.3 Narrow Band Imaging***

Endoscopes can now be built with narrow band imaging (NBI) red-green-blue filters. NBI uses the principle that different wavelengths of light penetrate the mucosa to different depths. Light of



short wavelength (blue light) penetrates only superficially with less scattering whereas light at longer wavelengths (red) penetrates more deeply but with lower resolution due to increased scatter.

Sharma et al reported preliminary results with NBI and magnification on 24 patients demonstrating that all patients with HGD in this series had both increased number of capillaries and an abnormal capillary pattern (either dilated, tortuous or corkscrew in appearance) (Sharma et al. 2003a). Subsequently a further study of 63 patients of whom 41 had HGD has identified three factors related to the presence of HGD. These factors include an initial impression of an irregular mucosal pattern under magnification white light endoscopy followed by the presence of one of the following two factors under NBI: either an irregular vascular pattern or the presence of abnormal blood vessels. All 41 patients had one of these three abnormalities described above and 85% had at least two present (Kara and Bergman 2006). Similar results were found by another group who studied 50 patients with BO using magnification endoscopy with NBI. These authors reported a sensitivity of 90% and specificity of 100% for the detection of HGD using the diagnostic criteria of irregular microvascular or microstructural patterns. Only 6 patients, however, in this study had HGD (Anagnostopoulos et al. 2007).

As described above a randomised crossover study has compared high resolution chromoendoscopy with indigo carmine staining to narrow band imaging demonstrating comparable sensitivities of 93% and 86% respectively in a small study of 28 patients, 14 of whom had HGD (Kara et al. 2005a).

A further advantage of NBI is that there is no need for staining agents. The principle drawbacks lie in the need for magnification, with detailed observation of the mucosa and then detailed inspection under NBI of suspicious areas. This not only requires expensive equipment and considerable expertise but also raises questions of inter-observer variability and the time necessary to complete the procedure. Some exponents suggest that AFI could be used to detect potentially suspicious areas and then NBI used in an immediately subsequent endoscopy to examine in more detail any suspicious areas. Kara et al have presented some limited data to suggest that this sequential technique may reduce the false positive biopsy rate for HGD to 10% while maintaining excellent sensitivity (Kara and Bergman 2006). This, however, is undeliverable to a surveillance population with Barrett's oesophagus with reference to cost and where clinicians

are already disheartened by a procedure time of 10-15 minutes for a return of about 1-3% of patients having HGD per endoscopy.

#### ***2.4.4 Optical Coherence Tomography***

Optical Coherence Tomography (OCT) is based on the same technique as endoscopic ultrasound (EUS) except that it measures the back-scattering of infrared photons instead of sound. It is a technique based on elastic scattering because there is no change in the wavelength of the back scattered light. OCT measures only singly scattered light (ie direct infrared light reflectance). This technique can provide tissue definition in the range of 1-10 micrometres with a penetration depth of 3-4 mm compared with the tissue definition of approximately 100 micrometres with the best currently available clinical high resolution EUS endoscope (Li et al. 2000; Tearney et al. 1997). Radial scanning is now also a possibility and it has been used successfully, producing good definition with in vitro specimens (Li et al. 2000). It has also been shown that varying the distance between the OCT probe and the oesophageal wall produces images of varying depth (Sivak, Jr. et al. 2000).

Jackle et al reported good resolution with OCT down to 10 micrometres and described distinct patterns of normal, inflammatory, premalignant and malignant tissues in the oesophagus (Jackle et al. 2000). One study of OCT in 32 patients demonstrated the ability of this technique to distinguish gastric mucosa from oesophageal mucosa, Barrett's oesophagus from normal oesophageal mucosa and oesophageal adenocarcinoma. Additionally, OCT provided clear delineation of layers of the normal human oesophagus extending from the epithelium to the longitudinal muscularis propria (Brand et al. 2000).

The ability of OCT to distinguish Barrett's oesophagus defined by the presence of specialised intestinal metaplasia was assessed in one study of 121 patients. Barrett's oesophagus was characterised by the absence of the layered structure of normal squamous epithelium, the presence of the vertical "pit and crypt" morphology of gastric mucosa with submucosal glands and the disorganised architecture of an irregular mucosal surface. OCT findings were compared to histology and Barrett's oesophagus was detected with a sensitivity of 97% and specificity of 92% (Poneros et al. 2001).

Only preliminary data has been reported regarding the use of OCT in detecting dysplasia in BO. One study of 33 patients compared OCT with histology for the detection of HGD and demonstrated a sensitivity of 68% and specificity of 82%. There was however very significant interobserver variability in diagnostic accuracy between the four observers ranging from 56% to 98% (Isenberg et al. 2005).

OCT may, therefore, have a potential role for the detection of dysplasia in Barrett's oesophagus but would require significant additional endoscopic skill and would require expert interpretation following the development of clear diagnostic criteria. OCT, at present, is more likely to be beneficial for the very accurate staging, down to 1 micrometre resolution, of malignant intramucosal oesophageal lesions and follow-up after therapy (Wong Kee Song and Wilson 2005).

## **2.5 Oesophageal Capsule in Screening for Barrett's Oesophagus**

The oesophageal capsule is a variant of capsule endoscopy which was first described in 2000 by Iddan et al. The oesophageal capsule collects a rapid sequence of images during swallowing and transmits these pictures via a radio transmitter for interpretation. Two initial studies have examined the cost effectiveness of the oesophageal capsule for screening the general population at age 50 for Barrett's Oesophagus or screening those with GORD symptoms. The first study aimed at screening white men at 50 years old estimated that OGD might prevent 60% of cancer deaths at a cost of \$11,254 per QALY gained compared to no screening. Capsule screening was estimated to prevent 53% of cancer deaths but was more expensive up to \$50,000 per QALY gained (Rubenstein et al. 2007). The second study also showed disappointing cost effectiveness when capsule was compared to OGD. The latter appeared cheaper and more effective in this study at \$1988 compared to \$2392 for the capsule for 18.54 and 18.36 life years gained respectively (Gerson and Lin 2007). The principle reasons for this lack of cost effectiveness are the relative poor sensitivity and specificity rates for detecting BO in patients with GORD. Sharma et al performed a study of 100 patients with reflux and found the capsule to have a sensitivity of 67% and specificity of 87%. These results translated to a PPV 60% and NPV of 90% (Sharma et al. 2007).

Consequently, at present, the capsule remains too inaccurate and therefore too expensive in screening for Barrett's Oesophagus. These problems with accuracy probably relate to either the

fast passage of the capsule through the distal oesophagus increasing the number of gastroscopies required due to incomplete images or the time required by experienced endoscopist to look through the images to diagnose patients with possible BO. One further blinded study examined the effectiveness of capsule compared to endoscopy in a surveillance population for the diagnosis of BO and showed a sensitivity and specificity of 67% and 84% respectively which translated to a positive and negative predictive value of 22% and 98% (Lin et al. 2007). The cost effectiveness of the capsule is then further limited by the current problems with surveillance which have been described above.

## **2.6 Non-endoscopic Immunocytological Screening for Barrett's Oesophagus**

One group from Cambridge have used a dissolvable capsule loaded with a sponge on a string as a potential non-endoscopic screening test for BO. The cytology that is recovered from the sponge can then be processed for immunological markers. In a preliminary study of 43 cases and 53 healthy controls immunocytology for Mcm2 had a sensitivity and specificity of 66% (Lao-Sirieix et al. 2007). The sampling device was well tolerated and generally acceptable to patients. As the authors correctly conclude the cytology which was acquired at relatively little cost and discomfort could be applied to other biomarkers of Barrett's Oesophagus that might perform better.

This is an exciting technique that in the future, with refinement of analysis, may yield an accurate screening test for the huge undiagnosed proportion of patients with BO in the general population.

## **2.7 Summary**

Novel endoscopic techniques are a rapidly moving branch of Gastroenterology and some of these are likely to be available outside a research setting within five years. The challenge for those involved in their development is to provide systems that are both affordable and improve diagnostic efficiency detecting more accurately those patients at high risk without the need for extended periods of further training. There are now new and interesting non-endoscopic techniques that may eventually allow for accurate primary screening for Barrett's in the general population. Efficient primary screening is almost a prerequisite requirement if the treatment of BO is to ever have a significant impact on overall rates of oesophageal adenocarcinoma in the whole population.

## Chapter 3

# Ablative Techniques for the treatment of Barrett's Oesophagus

### **3 Ablative Techniques for the treatment of Barrett's Oesophagus**

#### **3.1 Ablative Strategies for Barrett's Oesophagus**

Ablative therapies in BO can be directed at two different groups. The first is an attempt to eradicate all BO, both non-dysplastic and dysplastic, in all patients. The principle argument for this strategy is that BO confers an increased risk of oesophageal adenocarcinoma (OA) by 30-120x and surveillance is costly and fails to accurately predict those who will go on to develop cancer. The second main aim for therapy might be to direct treatment to those at highest risk of developing cancer and concentrate on the eradication of the high risk lesion (high grade dysplasia in BO at present). With this approach complete Barrett's ablation with squamous re-epithelialisation ('complete Barrett's ablation') might be considered an additional but not necessary goal.

##### ***3.1.1 Eradication of Barrett's Oesophagus***

While a laudable goal the complete eradication of all Barrett's in all diagnosed patients would be an ambitious objective. There are three central pillars required to make this a reality; high complete eradication rates for patients BO, buried Barrett's underneath re-epithelialised squamous mucosa (buried glands) should be close to, if not at zero, and a safe relatively cheap technique that can be used by the general gastroenterologist preferably as an outpatient. Other considerations must also include freeing the vast majority of treated patients from ongoing surveillance and there being a low recurrence rate of BO after successful ablation. The viability of complete ablation of BO for all patients potentially carries significant financial implications with health economics likely to become the overriding factor. The number of times it is necessary to perform this treatment and its subsequent cost potentially would prevent its introduction to the NHS even if all the criteria above were fulfilled.

##### ***3.1.2 Eradication of HGD***

The optimal treatment strategy for managing high-grade dysplasia (HGD) in BO is unclear. Oesophagectomy is conventionally considered as first-line therapy until recently because it has been the only reliable option for definitely removing all HGD and it was felt that the rate of concurrent but occult OA being present was approximately 50% (Heitmiller, 2003; Schnell et al,

2001). The real incidence is now probably lower with one of these groups analysing more recent data from 1994-2001 showing the harbored occult malignancy rate to have fallen to 17% (Tseng et al, 2003).

Oesophagectomy, even in high volume centres, has a 5-7.5% mortality and up to a 40% serious morbidity rate although in some centres a 0% operative mortality has been reported (Dimick et al, 2003; Dimick et al, 2005; Fernandez and Meyers, 2004; Hulscher et al, 2001; Keighley, 2003; Patti et al, 1998; Sihvo et al, 2004, Williams et al, 2007). Many patients are either unwilling or are elderly with co-morbid disease and considered unfit to undergo such a major procedure. Furthermore, several authors have reported Barrett's recurrence following oesophagectomy (Hamilton and Yardley, 1977; Lindahl et al, 1990; Oberg et al, 2002) with a more recent study of showing that 8/36 (22%) patients who have had subsequent endoscopies post surgery were found to have further BO (Wolfsen et al, 2004a). The authors of this study attribute Barrett's recurrence to uncontrolled acid reflux despite post-operative proton pump inhibition. Although acid reflux is more likely in this group, this does raise the question of recurrent BO forming after previous complete ablation with all ablative therapies including radiofrequency ablation.

Minimally invasive approaches for disease limited to the mucosa may be better and would almost certainly carry fewer risks. Mucosal ablation can be achieved by local destruction with thermal methods, freezing methods, light and chemical interaction in the presence of oxygen (photodynamic therapy, PDT) or physical removal by endoscopic mucosal resection (EMR).

**Table 3.1: Comparison of success rates of ablatable therapies:**

<b>Ablative Therapy</b>	<b>Dysplasia</b>	<b>Studies</b>	<b>Eradication of lesion</b>	<b>Cancer rate post therapy</b>	<b>Follow up</b>	<b>Comments and Complications</b>
<b>APC (&gt;60W)</b>	ND	4 Cohorts	72%-99%	NS	1-3yrs	Up to 30% Buried Glands, GI Bleeds, Perforations and Strictures reported
	HGD	1 Cohort	76%	14%	3yrs	1 Perforation (28 patients)
	ND	2 RCTs	93-97%	NS	6wks & 1yr	APC greater reversal than low dose ALA PDT in second of two RCTs
<b>RFA</b>	ND	3 Cohorts	90-98%	NS	<1yr	Short follow up (max 1 year)
	HGD	4 Cohorts	88-95%	NS	<1yr	Short follow up (max 1 year)
<b>EMR</b>	HGD	3 Cohorts	71%-95% (initial success)	NS	Up to 2yrs	10-19% GI Bleeds, Perforations Reported. 10-30% Metachronous lesions unless complete BO removal but this has 26-70% stricture rate
<b>PDT: Photofrin</b>	HGD	1 Cohort	78%	2%	4yrs	30% Strictures
	HGD	1 RCT	50%	14%	5yrs	33% Strictures, 33% photosensitivity 50% Significantly reduced cancer incidence
<b>PDT: ALA</b>	HGD	1 Cohort	89%	6%	3yrs	60mg/kg ALA. 785J/cm Light dose
	IMCA	1 Cohort	68%	32%	3yrs	No photosensitivity, No strictures

APC, Argon Plasma Coagulation;  
RFA, Radiofrequency Ablation;  
EMR, Endoscopic Mucosal Resection;  
PDT, Photodynamic therapy;

ND, Non dysplastic;  
HGD, High Grade Dysplasia;  
IMCA, Intramucosal Carcinoma;

NS, Not Stated;  
RCT, Randomised Controlled Trial;  
ALA, Aminolaevulinic acid;



### **3.2 Thermal Ablation of Barrett's Oesophagus**

Conceptually, the simplest endoscopic techniques for the ablation of HGD and early cancers are thermal (Sampliner, 1997).

#### **3.2.1 Argon Plasma Coagulation**

APC is a non-contact electrocoagulation using ionised argon gas. The APC probe is held a few millimeters from the target surface and involves using of a jet of argon gas to conduct an electric current to the target area resulting in coagulation. The depth of coagulation is up to a few millimetres. Of the thermal techniques the most easily performed, most extensively available and most studied is Argon Plasma Coagulation (APC).

Most studies of APC have been performed to assess its ability to effectively and safely treat non-dysplastic Barrett's oesophagus. There are many studies that report between 60-100% complete Barrett's ablation with subsequent re-epithelialised squamous mucosa. The effectiveness appeared to be dependent on the amount of energy delivered into the tissues (30-90W) (Attwood et al, 2003; Basu et al, 2002; Byrne et al, 1998; Kahaleh et al, 2002; Madisch et al, 2005; Manner et al, 2006; Morris et al, 2001; Pedrazzani et al, 2005; Pereira-Lima et al, 2000; Ragunath et al, 2005; Schulz et al, 2000). These eradication rates, however, come at a cost of both endoscopic time, with most patients requiring several treatments, and in complications. Some studies have seen significant gastrointestinal bleeding following therapy at higher powers (60-90W) at 4-9% (Dulai et al, 2005; Lantz and Vakil, 2003; Manner et al, 2006; Pedrazzani et al, 2005), others have noted stricture formation requiring dilatation in 4.3-9.1% (>65W) (Madisch et al, 2005; Morris et al, 2001; Pereira-Lima et al, 2000; Schulz et al, 2000) and oesophageal perforations have also been reported (Attwood et al, 2003; Byrne et al, 1998; Manner et al, 2006; Pereira-Lima et al, 2000).

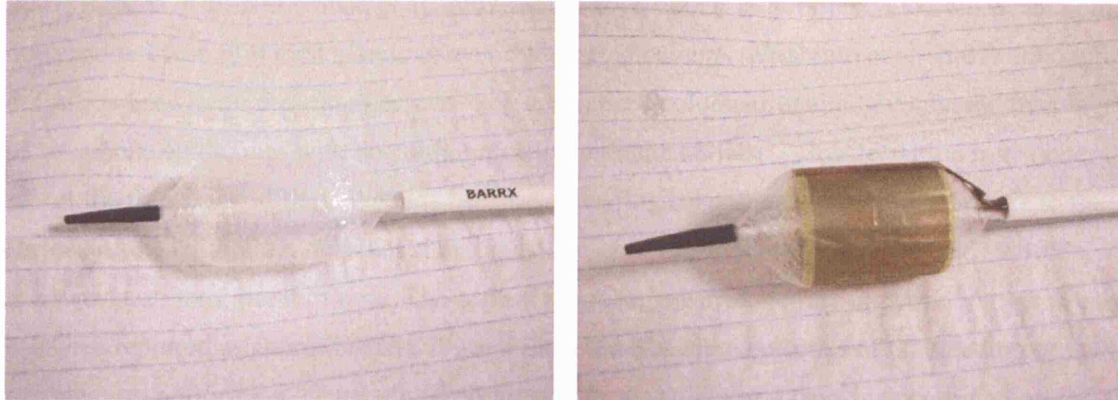
In one very safe study of 50 patients with very few side effects APC was performed at low power (30W) and there was less efficient complete ablation rates at 68% of patients with 44% of those having buried glands (Basu et al, 2002). This is of concern as in other studies patients have developed submucosal cancers during follow up (Kahaleh et al, 2002). The long-term efficacy of APC remains in question and one study suggests that the long-term eradication of BO is only 28% at 30months (Mork et al, 2007).

### ***3.2.2 Multipolar Electrocoagulation (MPEC) and Radiofrequency Ablation***

Multipolar electrocoagulation (MPEC) is a similar technique but used in contact with the mucosa and a coagulative effect is produced by passing a current between electrodes. Initial studies of MPEC were disappointing with the same principle limitations as APC. The first limitation is the difficulty in treating an extended area of BO due to discrete areas of burn; the second is the difficulty in controlling the depth of burn with too deep leading to stricture formation and too shallow incomplete ablation or development of buried glands. In studies treating non-dysplastic BO complete response occurred in 22-100% but buried gland formation occurred in up to 23% and the mean number of sessions up to 3.7 (Kovacs et al, 1999; Michopoulos et al, 1999; Montes et al, 1999; Sampliner et al, 2001; Sharma et al, 1999).

There is however a new generation of MPEC, radiofrequency ablation (RFA) for circumferential endoscopic ablation using an endoscopic balloon ablation device named HALO<sup>360</sup> from BarrX Inc. See figures 3.1 and 3.2. Sharma et al performed two preliminary studies on non-dysplastic BO; the first was a dosimetry study and the second an efficacy study. The dosimetry study used between 6, 8, 10 and 12J/cm<sup>2</sup> and each patient received up to two sessions of RFA. The complete Barrett's ablation rate (CR) was 0% with 6 and 8 J/ cm<sup>2</sup> and 30 and 36% for 10 and 12 J/cm<sup>2</sup>. The dosimetry chosen for the effectiveness study was 10J/cm<sup>2</sup> (performed twice during each endoscopy) for all patients. In the effectiveness study 70 patients were treated with relatively short BO (mean 3.2cm, range 2-6) and approximately 50% required a repeat double treatment at four months. A 69% complete response rate was achieved at 12months follow up (Sharma et al, 2007). There were no strictures, GI bleeds or buried glands in this study. This is very encouraging but in the treatment of non-dysplastic BO the goal of therapy must be complete ablation without the need for long-term follow up in order for this to be cost effective.

**Figure 3.1:** HALO 360 oesophageal sizing balloon and circumferential ablating device inflated with air



**Figure 3.2:** HALO 360 System with ablation balloon deflated prior to use



Subsequent studies with RFA have been more impressive. One study demonstrated that 1% N-acetylcysteine was an effective mucolytic agent and its use improved results (Pouw et al, 2007). This study also showed that accurately sizing the patient's oesophagus at either 22, 25, 28, 31 or 34mm with the sizing balloon in figure 3.1 above adequately flattened the oesophagus mucosa and ensured good contact with the electrodes without overstretching the oesophagus and risking stricture formation (Pouw et al, 2007). The sizing process has been automated and takes as little as five minutes. It has been shown that repeating the RFA treatment during each endoscopic procedure improves CR rates but does not increase the depth of ablation. For each 3cm of Barrett's, therefore, two treatments should be performed in one endoscopic session and any length of BO can be treated with 0.5-1cm overlaps (Dunkin et al, 2006). These overlaps have not been shown to increase the risk of stricture formation. In the treatment of dysplastic BO the advised energy level is  $12\text{J}/\text{cm}^2$  and for non-dysplastic BO  $10\text{J}/\text{cm}^2$ . The mean number of treatments was

1.4 with the 360 degree balloon and an additional 2.5 with the 90 degree balloon. Eradication rates for HGD were between 89-95% and complete reversal rates of BO 90-95% (Gondrie et al, 2007; Roorda et al, 2007; Shah et al, 2007; Smith et al, 2007). Additionally, there has been only one reported case of buried glands in over 600 treated patients (Mashimo et al, 2007) although the definition used in all these studies was "any specialised columnar epithelium covered by a layer of squamous epithelium with no communication with the surface". This definition may under-report the frequency of buried glands because a histological communication of Barrett's to the surface of the epithelium does not automatically ensure the endoscopist can see the Barrett's. This is however a minor point of note. The current oesophageal stricture rate in over 600 treated cases has been reported as approximately 1% and there have been no episodes of GI bleeding or oesophageal perforation. Chest discomfort lasted for a median of 4 days but prevented no patients from drinking adequately.

This novel technology and the recent results reported have appeared to leave other ablative technologies trailing in its wake. The studies to date however have been patient cohorts without any head-to-head comparisons and follow up has been short. It remains uncertain if these patients will be free of long-term surveillance and if a proportion will redevelop BO. Irrespective of this RFA has been shown to be simple to use, effective and safe for both non-dysplastic and dysplastic BO and is likely to play a major part in the treatment of BO in the future.

### ***3.2.3 Randomised Controlled Trials comparing thermal techniques***

There has been one randomised study comparing Barrett's reversal using APC to surveillance and PPI use. This was a limited feasibility study of only 40 patients with non-dysplastic BO. The follow up was for one year, too short a time to measure anything other than APC ability to reverse a Barrett's segment safely. In the APC arm 12/19 patients had complete reversal compared to 3/20 in the surveillance group (Ackroyd et al, 2004).

Two randomised studies have compared APC to multipolar electrocoagulation. In the first, 52 patients were studied and the results were not significantly different although there was a trend towards increased ablation histologically with MPEC (Dulai et al, 2005). There was, however, significant post-procedure bleeding at 8% after MPEC and 13% after APC. In the second study of 35 patients with non-dysplastic BO there was again no difference in the outcome between the groups. The rate of complete Barrett's reversal achieved was 75% with MPEC and 63% with

APC. This CR was only maintained in 70% of these patients at 2 years follow up with no predictors of who would relapse so that endoscopic surveillance had to continue to be offered (Sharma et al, 2006).

There have been four randomised studies that compared APC to other ablative modalities in BO. These are discussed in section 3.4 below.

### **3.3 Photodynamic therapy for Barrett's Oesophagus**

Photodynamic therapy (PDT) is the best-studied method for mucosal ablation in Barrett's oesophagus. It is a non-thermal, photochemical reaction resulting when light activates a photosensitiser in the presence of oxygen (Bown and Lovat, 2000). PDT has the advantage that light can be evenly and circumferentially distributed over the whole treatment area and is therefore particularly attractive for mucosal disease. PDT has been shown to spare collagen as tissue temperature remains unchanged preserving structural integrity and reducing the risk of perforation (Barr et al, 1987). PDT is therefore potentially an effective lower risk alternative to oesophagectomy.

Several photosensitizing agents have been used in the oesophagus including porfimer sodium (Photofrin), 5 aminolaevulinic acid (ALA) and meso-tetrahydroxyphenyl chlorine (mTHPC).

#### ***3.3.1 Photofrin PDT***

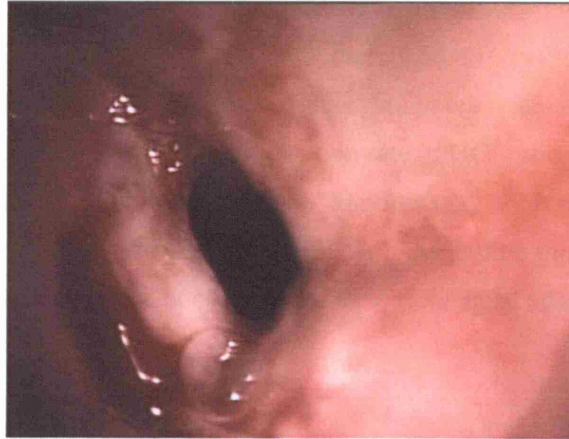
The first-generation photosensitiser, porfimer sodium or 'Photofrin' has been licensed in the UK and has recently been approved for use by the National Institute for Clinical Excellence (NICE) for the eradication of HGD in BO.

Overholt originally reported 87 patients treated with photofrin. Ten of 13 (77%) cancers were eradicated and HGD was cleared in 67/74 cases (91%) (Overholt et al, 1999).

Photofrin is taken up by both the mucosa and the submucosa and has, therefore, been associated with oesophageal stricture formation at a rate of 22% with one treatment and up to 50% with multiple treatments. See figure 3.4. These strictures often require multiple dilations (Panjehpour et al, 2005). Additionally, photosensitivity may last for as much as 90 days after treatment and reactions have been reported to typically occur in sun exposed areas on the hands, face or neck. This skin phototoxicity has been reported as mild in 66%, moderate in 3-24% and severe in approximately 1% (Overholt et al, 1999; Overholt et al, 2005). The incidence of severe reactions has been shown to vary widely and one study reported that 31% of cases required the prescription of oral steroids (Hemminger and Wolfsen, 2002; Wolfsen et al, 2004b). Many possible explanations for this variation exist, including the level of advice given to patients prior to discharge (especially the use of light metres), the rigor with which reactions were recorded,

whether patients were treated as inpatients or outpatients and in the definitions of severity involved. See figure 3.4 and 3.5.

**Figure 3.3:** Post Photofrin PDT Inflammatory Oesophageal Stricture



**Figure 3.4:** Severe skin photosensitivity occurring through clothing 1 month post Photofrin PDT taking six months to heal



**Figure 3.5:** Mild skin photosensitivity of the hand and chest occurring within first three weeks of photofrin PDT



A multicentre, international, randomised controlled trial confirmed the effectiveness of Photofrin PDT. Over 200 patients were recruited with HGD in BO and PDT was shown to reduce cancer rates by 50% in the PDT group when compared to patients treated with a proton pump inhibitor alone. Despite this good efficacy in this randomised study the success rate for the eradication of HGD appeared to fall at 5years follow up to approximately 50% (Overholt et al, 2005; Overholt et al, 2007). Additionally, there was still a 14% cancer rate in the treatment arm which may have been partly due to this lower than expected eradication rate of HGD and possibly due to the presence of occult tumours at randomisation. These tumours may not have been excluded because only a single endoscopy was performed prior to PDT with quadrantic biopsies every 2cm. It has been shown that there may be up to a 50% miss rate for oesophageal adenocarcinoma with this protocol (Reid et al, 2000).

An additional retrospective analysis of two cohorts of patients with HGD; one treated with Photofrin PDT and the other with oesophagectomy showed no significant difference in the survival of patients in either group. Perhaps the most serious criticism that could be levelled at this study was that all patients in the PDT group had previously seen an upper GI surgeon and either the patients or the surgeon decided that surgery was too high risk. The patients in the PDT group were consequently older with more co-morbidity (Prasad et al, 2007).

Two recent cost effectiveness analyses suggested that Photofrin PDT was the most cost effective option for treating HGD in BO when compared to oesophagectomy (Shaheen et al, 2004; Vij et al, 2004).

Photofrin is the most studied photosensitiser for the treatment of HGD in BO and has been shown to be effective not just for the eradication of HGD but for the much more robust and clinically relevant endpoint of cancer development. The lack of evidence for a reduction in the latter is often a criticism leveled at many studies of ablative therapy.

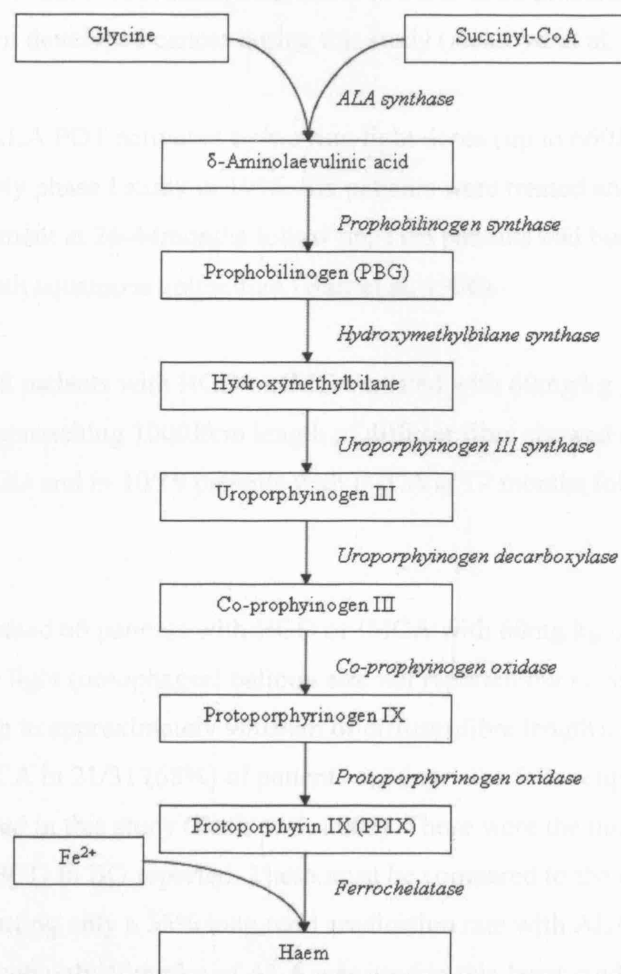
### **3.3.2 ALA PDT**

5-aminolaevulinic acid (ALA) is a naturally occurring substance in the haem biosynthesis pathway which is converted in vivo to its photoactive derivative protoporphyrin IX (PPIX). This is rapidly excreted resulting in skin photosensitivity only lasting 1-2 days. It has been estimated that the human body synthesises at least 358mg ALA per day to support endogenous



haem production. The control of this pathway and synthesis of ALA (the rate limiting step) is normally tightly regulated by feedback inhibition of ALA synthetase. When ALA is provided to the cell by exogenous drug administration this control point is bypassed and ALA enters the haem synthesis pathway unchecked, resulting in the accumulation of PPIX (Marcus et al, 1996). See figure 3.6. Furthermore, far more protoporphyrin IX is produced in the gastrointestinal mucosa than in the underlying muscle. The risk of stricture formation therefore has been shown to be minimal (Loh et al, 1996; Regula et al, 1995). These advantages, however, are offset against the PDT effect which has been demonstrated to be limited to a depth of 1-2mm, and ALA PDT has also been shown to be ineffective for invasive or nodular disease (Barr et al, 1996; Gossner et al, 1998). 5-aminolaevulinic acid (ALA) is therefore potentially a lower risk alternative to photofrin.

**Figure 3.6: Haem Biosynthesis Pathway**



Non-dysplastic Barrett's reversal has been assessed by one randomised controlled study of 25 patients using either 30mg/kg or 60mg/kg of ALA. There was a non-significant difference between the two groups using a moderate light dose of 668J/cm with an average of 60% Barrett's reversal (Kelty et al, 2004b). The ability of ALA PDT to completely reverse BO has been unconvincing and this along with its cost would prevent its use for the reversal of non-dysplastic BO. See table 3.2.

The ablation of dysplasia has been more extensively assessed. See table 3.2. Two studies have examined ALA PDT in the eradication of LGD in BO. The first was a randomised controlled study of 36 patients randomised to either PDT with 30mg/kg activated using a low green light dose of 264J/cm or PPI alone. ALA eradicated LGD in all patients (18/18) compared to 3/18 with PPI alone at 24 months follow up (Ackroyd et al, 2000). A cohort of 40 patients used the same parameters and has also shown successful treatment of LGD in 38 patients (95%) at 53months follow up. One patient developed cancer during this study (Ackroyd et al, 2003).

Barr et al first used ALA PDT activated by varying light doses (up to 660J/cm) in the ablation of HGD in BO in an early phase I study in 1996. Six patients were treated and none had residual HGD following treatment at 26-44months follow up. Two patients had buried columnar lined oesophagus underneath squamous epithelium (Barr et al, 1996).

In a larger study of 28 patients with HGD or IMCA treated with 60mg/kg ALA activated with a high red light dose approaching 1000J/cm length of diffuser fibre showed successful ablation in all 9 patients with HGD and in 10/19 patients with IMCA at 17 months follow up (Gossner et al, 1998).

The largest cohort treated 66 patients with HGD or IMCA with 60mg/kg of ALA activated by 150J/cm<sup>2</sup> of red laser light (oesophageal balloon size not reported but is believed to be 18mm which would translate to approximately 900J/cm of diffuser fibre length). HGD was eradicated in 31/35 (89%) and IMCA in 21/31 (68%) of patients at 36 months follow up. No serious complications occurred in this study (Pech et al, 2005). These were the most encouraging results for the treatment of HGD in BO reported. These must be compared to the disappointing results of a different study reporting only a 55% long term eradication rate with ALA PDT combined with EMR for HGD although only 40mg/kg of ALA was used in this latter study (Peters et al, 2005).

The PDT regimens used to treat dysplasia have varied in the amount of energy used to activate the photosensitiser (264J/cm-1000J/cm), the amount of oral ALA given prior to PDT (30-60mg/kg) and the wavelength of light used to activate the photosensitiser (red or green light). It is hardly surprising, therefore, that these studies have reported very different success rates for PDT. Furthermore, none of these studies have compared the relative efficacy of different doses of light in the same cohort of patients holding other parameters constant using the same enrolment criteria.

No oesophageal strictures or serious skin photosensitivity have been reported in all of these studies combined. Two deaths, however, have occurred following ALA PDT in two separate studies (Forcione et al, 2004; Haringsma et al, 2004). The cause of death was aspiration pneumonitis in one (Forcione et al, 2004) and not determined in the other, but was soon after treatment in a patient who was treated as a day case and may have been related to hypotension. As both incidents occurred following treatment as day cases this potentially affects the usefulness of ALA as day case procedure. Hypotension has been reported following oral ALA administration in doses of 60mg/kg in an inpatient study and was subsequently averted by giving intravenous fluids prior to ALA ingestion (Mackenzie et al, 2007). There has also been one case report of the development of peripheral neuropathy in a previously fit gentleman, similar to a porphyria like reaction, which gradually resolved following the administration of 60mg/kg (Sylantiev et al, 2005).

In summary ALA PDT may be a lower risk, more acceptable alternative to Photofrin PDT for HGD in BO. Studies are necessary to compare the effect of different parameters of ALA PDT prior to large randomised controlled trials comparing the efficacy of ALA to other photosensitisers (photofrin), other modalities (such as RFA) or surgery for the treatment of HGD in BO. ALA PDT does not seem to re-epithelialise a significant enough proportion of Barrett's to squamous mucosa to realistically be considered as an option for the treatment of non-dysplastic BO.

**Table 3.2: Studies to date with ALA PDT for Barrett's Oesophagus**

Author, Year	n	Histology	Aim	ALA Dose mg/kg	Light Dose J/cm	Red or Green	Outcome	Comments
Hage, 2004	26	No dysplasia	RCT PDT vs APC BO Reversal	60	785 +/- Fractionated	Red	PDT 82% Fractionated 90% APC 67%	Follow up 12m 1 Death
Kelty, 2004	34	No dysplasia	RCT vs APC BO Reversal	30	668	Red	Equal 50% Ablation	24% Buried Glands
Kelty, 2004	25	No dysplasia	RCT 30 v 60 BO Reversal	30 v 60	668	Red	No difference	PPIX level NS different
Ackroyd, 2003	40	LGD	Cohort, LGD Eradication	30	264	Green	38/40. (95%) Eradication LGD	53 months follow up
Ackroyd, 2000	36	LGD	RCT PDT vs PPI	30 v PPI	264	Green	Success: PDT 18/18(100%) PPI 6/18 (33%)	
Barr, 1996	6	HGD	Variable Light Dose	60	Max 660	Red	6/6 Success	2/6 Buried Glands
Peters, 2005	33	HGD/ IMCA	EMR then 20 PDT	40	785	Red	11/20 (55%) PDT Success	
Forcione 2004	6	HGD/ IMCA	Eradicate lesion	30	150 J/cm <sup>2</sup> *	Red	(1/5) 20% Success	1 Death
Gossner, 1998	28	HGD/ IMCA	Eradicate lesion	60	948	Red	Success HGD 9/9 IMCA 10/19	17 months follow up
Pech, 2005	66	HGD/ IMCA	Eradicate lesion	60	150 J/cm <sup>2</sup> *	Red	Success HGD 31/35 (89%) IMCA 21/31 (68%)	60 months Follow up

\* No balloon size reported to calculate energy into the tissues

### 3.3.3 Foscan PDT

m-tetrahydroxyphenyl chlorine (mTHPC) is a powerful photosensitiser approved in Europe for the palliative treatment of advanced head and neck cancers. The evidence for its use for HGD or localised adenocarcinoma in BO is very limited. Skin photosensitivity still lasts for 2-3 weeks and there is some risk of stricture formation because mTHPC is still mainly found in the submucosa although more drug is found in the mucosa than with porfimer sodium (Mlkvy et al, 1998).

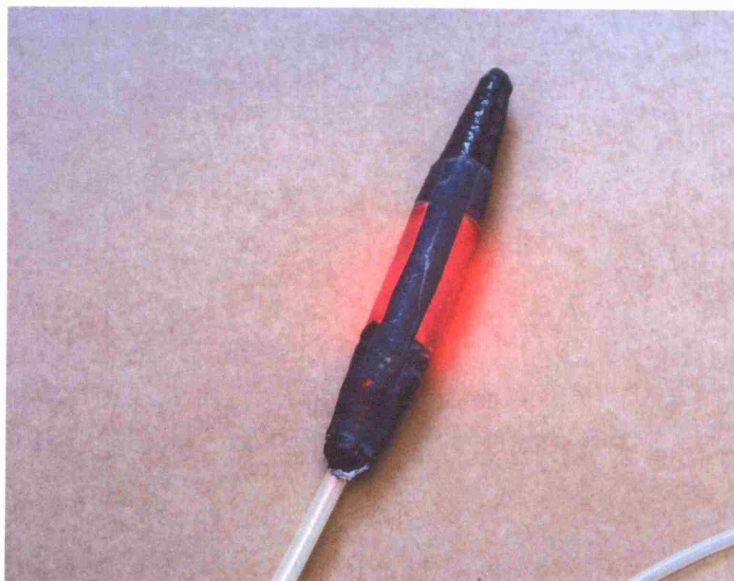
Javaid et al in 2002 successfully treated 5 out of 7 patients with HGD or IMCA at a median follow up of 1 year.(Javaid et al, 2002) Another study reported successfully treating 12/12 patients with HGD or early OA using green light to activate mTHPC for PDT with a median follow-up of 18 months. Just one patient required further PDT and this was successful (Etienne et al, 2004). These results contrast significantly with a study which used both green and red light. In

this study the best results using mTHPC-PDT were achieved with red light activation despite 90° of the silicone bolster being blacked out preventing mucosal damage occurring to that proportion of the oesophagus (figure 3.5). The use of this regimen resulted in 7/9 patients in long-term remission with PDT alone including two T2N0 invasive adenocarcinomas (figures 3.6 and 3.7) compared to 0/3 treated with green light (Lovat et al, 2005). The only apparent difference was in the drug-light interval. Etienne et al used an interval of four days compared to Lovat et al who used 3 days. Although pharmacokinetic data for mTHPC in human oesophageal tissue suggested an optimal drug-light interval of either 3 or 4 days (Andrejevic et al, 2001) further consideration of this interval is required.

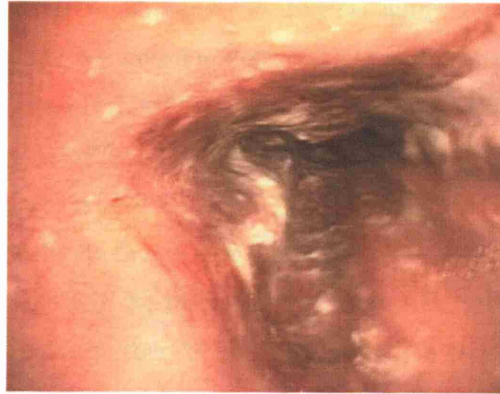
Of concern with mTHPC PDT was the report of two oesophageal perforations although the authors attributed these to early biopsy following therapy which would now not be advised. Additionally, in one of the two cases a very high light dose was given to a single area which is also now not recommended (Lovat et al, 2005).

The data regarding mTHPC use for BO is minimal and requires further study but the powerful nature of the photosensitiser with short light activation and the possibility of treating T2 cancers is interesting.

**Figure 3.7:** Silicone bolster used at UCH with 90 degrees of the circumference blacked out to reduce the risk of stricture formation



**Figure 3.8:** One day post Foscan PDT with a 90 degree blacked out area. The right wall is subsequently spared from necrosis



**Figure 3.9:** Oesophagus healed 8 weeks after Foscan PDT. Complete Barrett's reversal in the treated area with 'strip' of residual Barrett's along the right wall



### **3.4 Randomised Comparative Trials between APC and PDT for Barrett's reversal**

Two randomised studies have compared APC to PDT with the primary endpoint being Barrett's re-epithelisation to squamous mucosa in non-dysplastic BO. The first examined the effectiveness of Barrett's ablation between ALA PDT (60mg/kg ALA, 785J/cm of diffuser fibre at 4 hours), fractionated light dose ALA PDT (155J/cm at one hour then 785J/cm at 4hours) and APC (65W) in 40 patients. Fractionated ALA PDT and APC significantly reversed a greater percentage of the BO 86% and 93% respectively than single light dose ALA PDT (51%). Fewer patients had complete ablation at 33-36% in the fractionated PDT and APC groups. Twenty-three out of forty patients however did receive further APC which resulted in complete ablation 27/33 patients still in follow up (Hage et al, 2004b).

A second trial randomised 68 patients to either ALA PDT at 30mg/kg using red light or APC at 65W. Macroscopic ablation of BO was greatest in the APC group at 97% (33/34) but 21% had buried glands. In the PDT group there was a complete Barrett's reversal in only 50% of cases and again the incidence of buried glands were high at 24% (Kelty et al, 2004b).

One study has randomised 26 patients with dysplastic BO to either Photofrin PDT using 2mg/kg photofrin activated with 200J/cm red light in a single session or APC at 65W treated over 1-6 sessions. Most patients had LGD (23 patients) not HGD (3 patients). At 1 year follow up APC had reduced the length of Barrett's by 56% and PDT by 60% with dysplasia eradicated in only 67% and 77% respectively. Buried glands were seen in both groups and one patient developed a sub-squamous cancer in the PDT group. Furthermore, there was a 15% stricture rate in both groups as well as 15% of patients in the PDT group suffering from photosensitivity reactions (Ragunath et al, 2005).

None of these studies would support the use of either APC or PDT where the primary aim was the complete reversal of non dysplastic BO to squamous mucosa.

### **3.5 Endoscopic Mucosal Resection**

Endoscopic mucosal resection (EMR), until recently with the introduction of the multiband ligator, either required the submucosal plane to be injected with fluid to 'lift' the mucosa off the

muscularis mucosae underneath or the lesion to be 'sucked' into an EMR cap. The abnormal area was then snared and removed by electrocautery similar to the removal of a colonic polyp. EMR is an attractive option, particularly for well-defined lesions, limited to the mucosa.

The first large study of 64 patients who had oesophageal neoplasia (HGD or IMCA) resected by EMR when abnormalities were visible was reported by Ell et al in 2000. The authors performed a total of 120 resections with 97% complete resection in the most localised lesions compared to 59% for the larger intramucosal cancers. Recurrent or metachronous carcinomas, however, were found in 14% during the short 12months follow up (Ell et al, 2000).

Neoplastic lesions in BO may not always be visible and are often patchy and occult. These lesions are not easily detected and subsequently it is harder to target the lesion for EMR. Several studies, however, have attempted resections in these patients.

Three of these studies examined EMR alone for this purpose. Two of the studies which aimed to resect only the occult neoplastic area achieved complete initial resection in between 71% (15/21) and 92% (36/39) but two patients had local recurrence in the former study. Gastrointestinal bleeding however complicated therapy in 19% and 10% respectively (Conio et al, 2005; Giovannini et al, 2004). The third study was more ambitious with 39 consecutive patients treated with the aim of removing all of the Barrett's mucosa in addition to the area of neoplasia. Two patients did not complete therapy due to other co-morbidity. Of the 37 patients completing treatment four required additional therapy with APC but all had complete eradication of HGD or IMCA and 89% complete removal of all BO. These results, however, came with an increased complication rate with one perforation, one late gastrointestinal bleed and 10/36 (26%) developing symptomatic strictures (Peters et al, 2006).

Four studies have examined combination ablative therapies for the treatment of neoplastic lesions and used EMR for visible lesions with PDT for residual flat HGD or PDT alone for non-visible lesions. In two of these studies the ablative therapy group as a whole achieved good initial complete remission rates in 98% of patients in each study (43/44 and 113/115). These combination therapies were safe with few complications with just 3 stenoses following EMR. There was however a metachronous recurrence rate of HGD and IMCA of between 17% and 30% (Behrens et al, 2005; May et al, 2002). The third study evaluated minimally invasive endoscopic therapy in 33 patients referred for the management of early neoplasia in BO. All had initial EMR

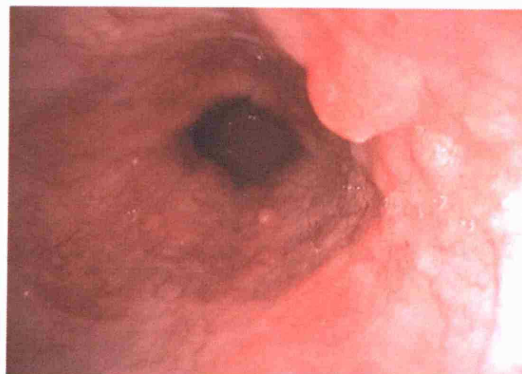


but five immediately went on to oesophagectomy either due to disease not being confined to the mucosa or due to patient choice (one patient). Of the remaining 28 patients: 18 had further EMR, 19 received ALA PDT and 3 had APC. Ninety three percent (26/28) had initial successful therapy but five of these required further EMR which successfully treated the residual neoplasia. All 26 patients who had initially successful endoscopic therapy and 93% in total were in local remission at a median follow up of 19 months (Peters et al, 2005).

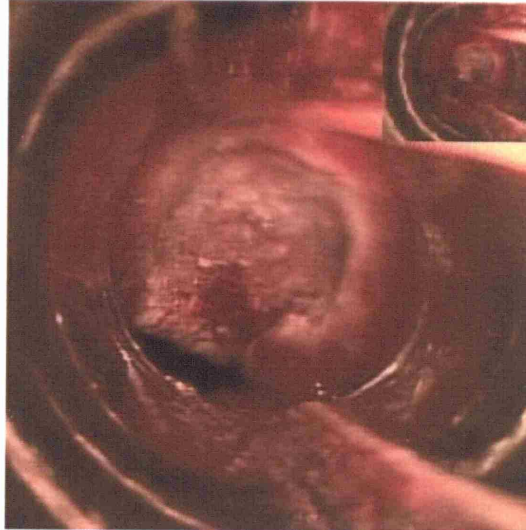
The final study of 88 patients compared oesophagectomy to EMR and PDT in a retrospective review over a five year period. Unsurprisingly the oesophagectomy group had a significantly high procedure related complication rate at 31% including one death compared to the complication rate in the ablative therapies group of just 4%. Twenty out of 24 patients in the ablative therapies group remained free of cancer over the relatively short follow up period of 1 year compared to all patients in the surgery group. Of the four patients relapsing two had successful repeat therapy and two died of unrelated causes. The authors reasonably concluded that ablatives are a safer alternative to oesophagectomy with good cure rates and suggest a randomised controlled trial of ablative therapies compared to oesophagectomy (Pacifico et al, 2003).

One feasibility study aimed to assess a new multiband variceal ligator (figure 3.9 and figure 3.10) for circumferential EMR aiming at complete BO removal. Although successful in 9/10 patients seven developed strictures with particular risk identified if circumferential EMR was performed in a single session (Soehendra et al, 2006). A second study examining circumferential EMR retrospectively analysed 41 patients with either HGD or early cancer (51%) with a mean follow up of 32 months. Seventy six percent had complete BO reversal although this dropped to 50% during follow up. Complications however were common with 8 cases of bleeding, 2 perforations and one oesophageal stricture. Two patients developed recurrent HGD and 7 had recurrent or new intramucosal cancer (22% relapse rate) although most were successfully retreated with EMR.

**Figure 3.10:** Nodule of HGD in BO amenable to EMR

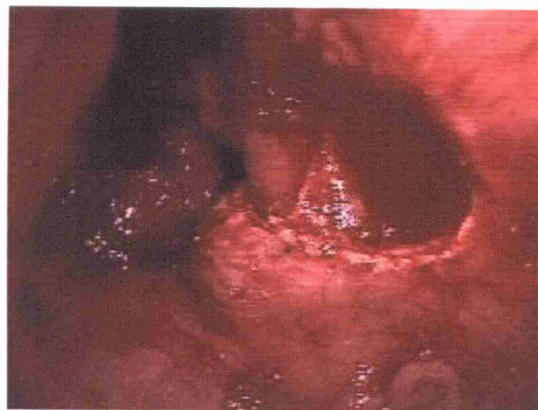


**Figure 3.11:** Post EMR view through multiband ligator device.



Ulcer base completely exposed post EMR with muscularis mucosae exposed and no post resection bleeding

**Figure 3.12:** Post EMR Bleed



Multiband EMR was compared to historical matched controls for safety and efficacy showing a 6% GI bleeding rate (figure 3.11) compared to a 20% rate for cap EMR. Although the EMR specimens with the multiband EMR device were slightly smaller (17mm vs 21mm) the time taken to perform an EMR was half at 6 minutes (Peters et al, 2007).

In summary, EMR requires a high degree of endoscopic skill and is associated with bleeding at the resection site in up to 20% of patients although this is rarely life-threatening haemorrhage. It does, however, seem extremely effective in the treatment of visible areas of neoplasia in BO. Circumferential EMR may also be successful but is more controversial (Katada et al, 2003; Seewald et al, 2003). It is more technically demanding, still carries the risk of gastrointestinal bleeding, can result in oesophageal stricture formation in up to 70% of cases and can rarely result in perforations (Peters et al, 2006; Satodate et al, 2004; Soehendra et al, 2006). It may be that there are other safer and less technically demanding alternatives to treat an area of flat dysplastic BO such as PDT or RFA. Finally, unless the whole segment is successfully treated then the occurrence of metachronous lesions is high between 10% and 30% of cases (Behrens et al, 2005; Giovannini et al, 2004; Lopes et al, 2007; May et al, 2002).

### **3.6 Cryotherapy with Liquid Nitrogen**

Early proof of concept studies have been performed using sprayed liquid nitrogen onto the oesophageal mucosa via a low pressure spraying catheter placed through the biopsy channel of an endoscope under direct vision. This technique has been shown to completely reverse Barrett's mucosa in 64% (7/11) with only a few buried glands demonstrated (<0.75%) at 12 months follow up. Additionally in the six patients with low or high grade dysplasia proceeding treatment none had any dysplasia found post therapy. The mean number of sessions required to achieve these results however was 4.6.(Johnston et al, 2005). No GI bleeds or oesophageal strictures were reported. A subsequent study of 14 patients with HGD and IMCA in BO were treated with cryotherapy and 9/10 patients (90%) with HGD and 3/4 patients (75%) with IMCA had their neoplastic lesions eradicated in a median of 3.5 sessions. There were, however, some significant side effects including 3 oesophageal strictures (one requiring dilation) and one gastric perforation in a patient with Marfan's syndrome (Dumot et al, 2007).

Cryotherapy is an exciting concept which has been extensively used elsewhere in the body especially by dermatologists for skin lesions. If successful it offers a cheap and easy to use way of ablating BO. Ultimately the success of this therapy will lie in the delivery device allowing for easy, uniform delivery of liquid nitrogen and a subsequent reduction in the large number of repeat endoscopic sessions. Long-term safety and efficacy data in larger groups of patients are required.

### **3.7 Residual genetic abnormalities and residual Barrett's Oesophagus underneath re-epithelialised squamous mucosa**

All ablative techniques are associated with the occurrence of buried Barrett's epithelium underneath squamous mucosa following therapy (Ban et al, 2004; Basu et al, 2002; Kelty et al, 2004b; Mashimo et al, 2007). These buried glands are a concern as subsquamous adenocarcinomas have been reported (Overholt et al, 2003; Overholt et al, 2005; Shand et al, 2001; Van Laethem et al, 2000). The frequency that these occur may be related to the treatment parameters used. For example, in a study that used low power APC (30W) the incidence of buried glands was as high as 44% (Basu et al, 2002) compared to other studies using higher power reporting rates of 21% (Kelty et al, 2004b). In an ALA PDT study using lower light and drug doses a high rate of buried glands was also seen 24% (Kelty et al, 2004a). Minimising the occurrence of these 'buried glands' would, therefore, be desirable.

Additionally in residual Barrett's epithelium persistent genetic abnormalities may be present. Krishnadath et al in 2000 examined biopsies from three patients who developed HGD during follow up after apparent eradication. All three could be shown to have residual genetic abnormalities following PDT but the numbers were small and there was no control group. The genetic abnormalities present showed one with aneuploidy and one with tetraploidy. Both cases also displayed increased p53 expression. The third case had p16 methylation and a p53 mutation (Krishnadath et al, 2000). Aneuploidy, tetraploidy and p53 mutation are the only biomarkers to have reached phase 4 of biomarker development and again seem to predict future risk of neoplastic development. This study suggested that histological examination alone for the detection of dysplasia was not enough to exclude future cancer risk. A second study has shown genetic abnormalities in residual glandular epithelium at the new squamocolumnar junction with 13/21 (62%) having increased Ki-67 staining indicating increased proliferation, 8/21 (38%) demonstrating increased COX-2 expression and 8/21 (38%) showing increased p53 staining (Dvorak et al, 2006). Two other studies have examined the residual Barrett's segment following ablative therapy. Both have shown that genetic abnormalities may remain if present before treatment but these have not been associated with future risk of either HGD or cancer (Hage et al, 2004a; Hage et al, 2006). These studies are important because they have suggested a potential reason for late relapse following PDT in the absence of residual dysplasia and a possible way of identifying these cases.

### 3.8 Summary

There remains no clear cut 'best' alternative for the management of BO.

Modalities, at present, for the ablation of a non-dysplastic Barrett's segment are prohibitively time consuming, often leave residual BO and therefore all patients currently require follow up. Ultimately, this is unlikely to be cost effective. Any ablative therapy aimed at all patients with BO would have to be cheap, fast, safe and not prone to leaving residual Barrett's behind risking future malignant change.

The risk-benefit balance for the treatment of HGD and IMCA however is different in favour of ablative therapies. All current treatments for dysplasia in BO run the risk of leaving residual BO with genetic abnormalities and malignant potential or BO redevelopment and therefore patients require ongoing surveillance.

The debate regarding the use of surgery or minimally invasive techniques for the treatment of neoplasia will rumble on. As suggested by Pacifico et al a randomised controlled trial of oesophagectomy versus ablative therapy may be the next step and this would be a ground-breaking piece of work. There remain several significant barriers, however, to the inception of such a study. The first is that this would almost certainly have to be multicentre and would require the agreement of a group of committed and interested surgeons and an equally skilled group of minimally invasive endoscopists either on the same site or nearby to allow effective randomisation. The second is deciding on the most effective and safest ablative therapy regimen for comparison, such as EMR for visible lesions and PDT or RFA for any residual HGD, in what is becoming a rapidly moving therapeutic area. It may be that this trial of oesophagectomy compared to a minimally invasive therapy is no longer viable with respect to patients agreeing to be randomised or ethical with very low risks now being reported with both ALA PDT and RFA.

## Chapter 4

# Aims of this thesis

#### **4 Aims of this Thesis**

The overarching aim of this thesis is to develop novel technologies to improve both the diagnostic efficiency and more accurately define those patients at high risk in Barrett's Oesophagus. It is then important to be able to offer patients a minimally invasive, lower risk alternative to oesophagectomy.

More specifically, diagnostic accuracy may be improved if the detection of aneuploidy could be introduced into routine clinical practice and combined with HGD with around 87% of patients not having aneuploidy on a series of posterior biopsies collected at 2cm intervals through their Barrett's segment. If they also were free of HGD then the earliest these patients may require repeat endoscopy could be as long as 5 years or more. From the perspective of screening and secondary surveillance, many older patients may not require a second endoscopy at all following an initial screening endoscopy with patients, clinicians and health economists accepting a small 'miss rate' for future cancer development.

Flow cytometry, the current method of detection of aneuploidy is laborious, time consuming, error-prone and difficult to reproduce outside of one or two research centres. Image cytometry while remaining time-consuming and laborious would appear to be less error-prone. Flow and Image cytometry are, however, both unlikely to be translated into routine clinical practice unless major automation of the process occurs.

The first major part to this thesis deals with improved methods of predicting future cancer risk in Barrett's oesophagus. The principle aims for this include:

- The validation of Image Cytometry in the prediction of future cancer risk in BO, and
- The development of Elastic Scattering Spectroscopy (ESS) to target biopsies for aneuploidy and high grade dysplasia.

The validation of image cytometry falls into several key areas. First is the ability of the image cytometry machine to accurately record the DNA content of Barrett's mucosal cells. Many factors may affect this measurement such as processing a cut section from a paraffin block, the number of nuclei collected for histogram analysis and if the image cytometry machine measures the same DNA content as flow cytometry. Secondly, the predictive ability of aneuploidy for future cancer risk measured using image cytometry will be validated by performing a case control study of the

presence of aneuploidy prior to the recurrence of HGD or development of oesophageal adenocarcinoma in patients treated with PDT.

The second major part to this thesis is the examination of photodynamic therapy as a minimally invasive low risk alternative to oesophagectomy. Two drugs have become the major focus of research work in the treatment of HGD in Barrett's Oesophagus. This work examines the best parameters of ALA-PDT and then compares this optimal regimen of ALA-PDT to Photofrin PDT currently the minimally invasive therapy of choice for HGD in BO.

Surveillance for Barrett's Oesophagus remains controversial and at present is time consuming, inaccurate and expensive. If patients are found to be at high risk in these programs then the vast majority at present will only be offered surgery with all of its adherent risks. This project aims to advance our knowledge in both the diagnostics and minimally invasive treatment of BO.



## Chapter 5

# Image Cytometry for the Detection of Aneuploidy in Barrett's Oesophagus

## 5 Image Cytometry for the Detection of Aneuploidy in Barrett's Oesophagus

### 5.1 Introduction

#### *Aneuploidy and future cancer risk*

When considering a population of cells with acquired aneuploidy measured by image or flow cytometry aneuploidy can be defined as an alteration in total nuclear DNA content. This normally manifests itself as an increase in nuclear size and density. Aneuploidy has been shown to precede the development of cancer and has been shown to predict future cancer risk in oesophageal, breast, lung, cervical and colorectal cancer (Fabian et al, 1994; Grote et al, 2004; Reid et al, 2000; Sjoqvist et al, 2005; Smith et al, 1996). Additionally it has been associated with a worse prognosis and earlier recurrence in breast, colorectal and lung cancer (Kasprzyk et al, 2006; Lanza et al, 1998; Moureau-Zabotto et al, 2005). One general definition of aneuploidy produced by the European Society of Analytical Cellular Pathology is:

*“In cytometry, aneuploidy is used to describe a change in the overall DNA content. Diploid cells have a DNA content of normal cells although their chromosomes may be abnormal.”*

European Society for Analytical Cellular Pathology (ESACP) guidelines.  
(Bocking et al, 1995)

More specifically, the most advanced evidence for aneuploidy defining future cancer risk in Barrett's Oesophagus relates specifically to changes in the overall total quantity of DNA in the nuclei of a group of cells in the study population. In truth this is a rather crude measure of alteration and as mentioned in the definition above this makes no judgment as to the normality of each chromosome or gene within that nucleus. Furthermore the definitions for flow cytometric abnormalities as defined by the Seattle group only look at the proportion of abnormal nuclei not just presence or absence of any given genetic defect. As stated in chapter 1, it may now be possible to predict more accurately an individual's risk of developing cancer in Barrett's Oesophagus. Three groups, two using flow cytometry and one using image cytometry, have confirmed that aneuploidy predicts future cancer risk (Fang et al, 2004; Rabinovitch et al, 2001; Reid et al, 2000; Reid et al, 2001; Teodori et al, 1998). The largest of these studies is a prospective cohort study of 322 patients defining future cancer risk and was performed by using flow cytometry. This study has identified the importance of a, difficult to measure, subset of

aneuploidy termed tetraploidy. Tetraploidy is the proportion of nuclei in the biopsy specimen with almost exactly twice the DNA copy number (4N fraction, 3.85-4.1N). In Seattle's early studies from 2000 and 2001, prior to cell sorting techniques all cells with a 4N DNA complement were termed as the tetraploid fraction. This subgroup is important because its detection significantly improves prediction of future cancer risk with up to 40% of patients having tetraploidy as the sole flow cytometric abnormality (Reid et al, 2001). This group also examined the significance of the proportion of cells undergoing DNA synthesis as part of cell division (S Phase fraction). They found that greater than 5.5% of cells as an S phase fraction predicted a future cancer risk of 21% at five years in a univariate analysis. Once aneuploidy and tetraploidy were included in a multivariate analysis, however, S Phase fraction was no longer significant (Rabinovitch et al, 2001).

The flow cytometric abnormalities relating to aneuploidy and tetraploidy have been defined by this group as:

Aneuploidy is a second peak distinct from the diploid peak of greater than 2.5% of the nuclei in a population of cells.

Tetraploidy should be considered present if the 4N peak (3.85-4.1N) is above 6% of the total proportion of cells.

(Vaughan et al, 2005)

Total nuclear DNA may be altered by changes in the size or number of chromosomes by replication, polyploidisation, gain or deletion. Aneuploidy is often associated with a p53 mutation. One effect of a p53 mutation is to allow cells with genetic abnormalities to more easily enter mitosis (Bocking et al, 1995; Rabinovitch et al, 2001). p53 abnormalities are closely associated with the subsequent development of aneuploidy and the majority of patients with aneuploidy have a preceding p53 mutation. Although p53 is responsible for one of the checkpoints preventing cells with genetic abnormalities entering mitosis, p53 abnormalities are not a prerequisite to the development of aneuploidy as a small number of cases exist without this mutation (Reid et al, 2001).

### ***Image Cytometry for the measurement of aneuploidy in BO***

The most widely validated method for the detection of aneuploidy has been flow cytometry. Flow cytometry can be used to examine, sort and count particles. In relation to aneuploidy the nuclei are stained (depending on the wavelength of the exciting laser, for example, with propidium

iodide) and suspended in phosphate buffered saline (PBS). The nuclei are then hydro-dynamically focused into the centre of the sheath by an additional flow of PBS and past a laser beam of light. This laser light is specifically focused onto the centre of the sheath and shone at a wavelength designed to excite the nuclei (488nm for propidium iodide). The nuclei excited by this light then emit light at a different wavelength (fluorescence). Flow cytometry is able to measure this fluorescence for each individual nucleus allowing for a quantitative measure to be made for each individual nucleus.

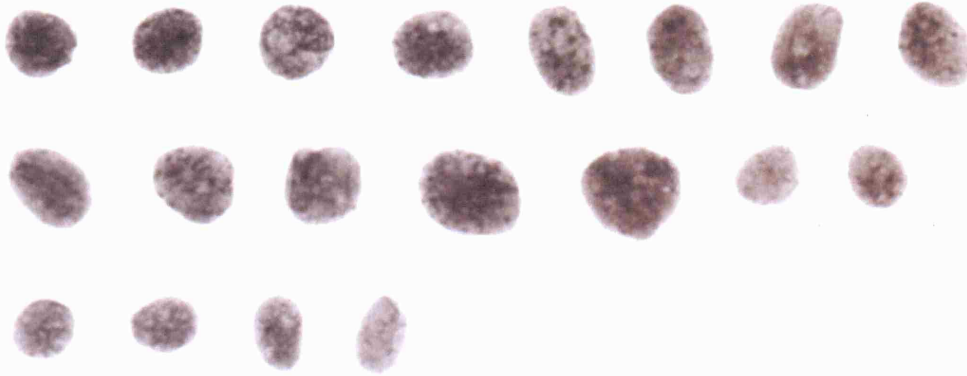
Flow cytometry, however, is costly and labour intensive. Additionally, unless the most rigorous methodology is used flow cytometry can result in false positive errors either due to lysis resulting in the appearance of a near diploid aneuploidy or nuclear clumping resulting in an increased 4N fraction (Alanen et al, 1989; Reid et al, 1992). These limitations, therefore, make flow cytometry impractical for routine clinical use.

A newer method for detecting aneuploidy *ex vivo* relies on image cytometry. Image cytometry accurately determines the amount of nuclear DNA by using a Feulgen stain and subsequent green light absorption. Feulgen staining specifically identifies DNA or chromosomal material in the specimen because it will only stain aldehydes. After cytoplasm digestion the only aldehydes remaining are those from the hydrolysis of DNA (by HCl). A quantitative measure of the DNA in the nucleus can then be calculated using a digital microscope and high pixel digital camera. Feulgen stains DNA red and with green light shone from beneath the slide into the microscope the digital camera measures the absorption of this green light by the nuclei under investigation relative to the background light intensity.

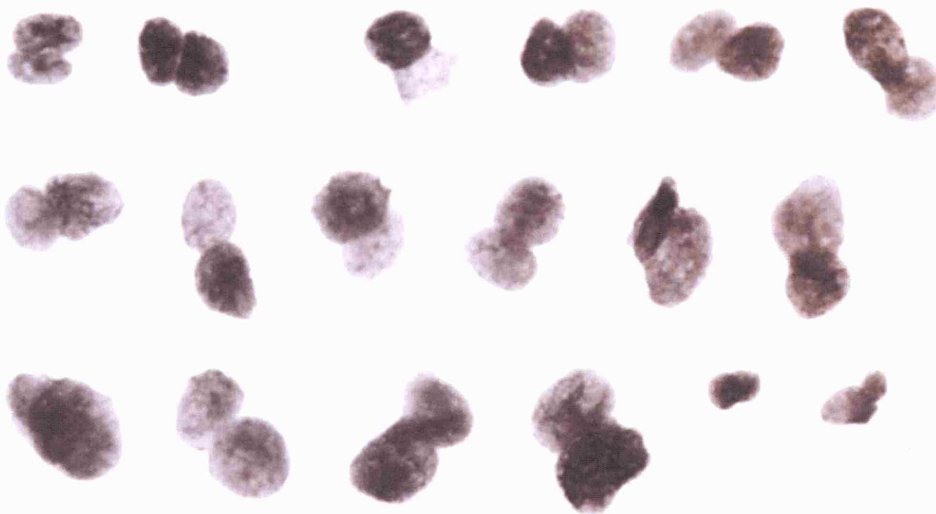
A picture of each individual nucleus was collected automatically by the image cytometry machine. The machine identified each nucleus on the slide against the mean background intensity of light. Using a range of pre-defined criteria relating to the physical properties of the nuclei including total area, symmetry, eccentricity, and optical density the image cytometry machine selected nuclei. These 'rules' for nuclei selection from the slide were kept deliberately broad which resulted in the collection of a few additional incorrect or malformed nuclei but ensured no nuclei were 'missed' on account of being very large or 'oddly' shaped. Manual exclusion of non-nuclear fragments, cut nuclei or doublets were made on viewing the presented galleries all of the collected nuclei. See Figure 5.1 for examples of included and excluded nuclei and Figure 5.2 of the presented galleries prior to analysis.

**Figure 5.1:** Examples of nuclei included in the analysis and those excluded as cut or multiple adjoined nuclei

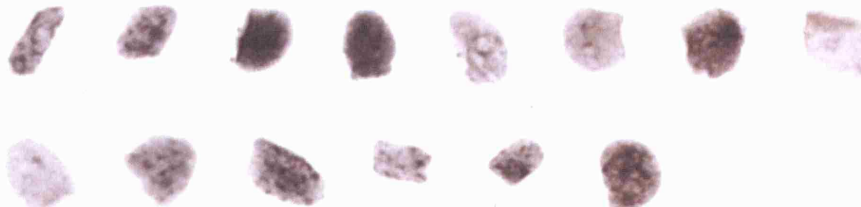
**Figure 5.1a)** Mixture of well formed diploid and aneuploid nuclei later included in ICM analysis



**Figure 5.1b)** Examples of multiple nuclei in clumps excluded from analysis (same scale as 5.1a)

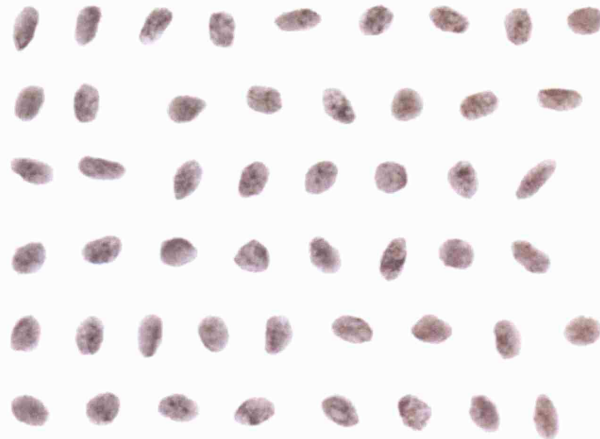


**Figure 5.1c)** Examples of cut nuclei excluded from analysis (same scale as 5.1a)

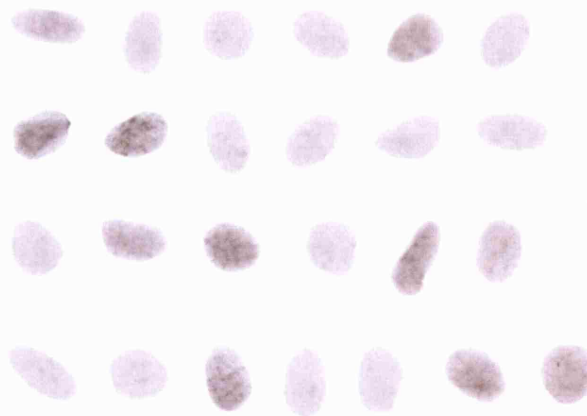


**Figure 5.2: Example of Image Cytometry Galleries**

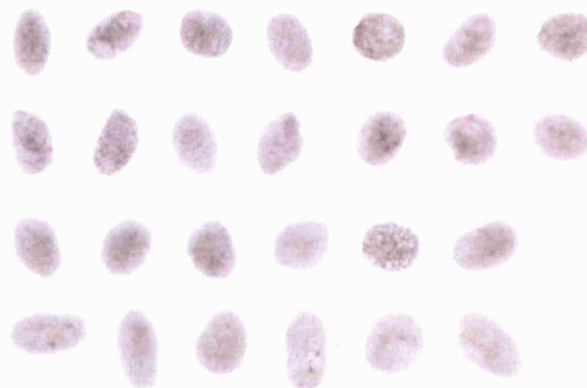
**5.2a) Gallery of Diploid Nuclei on later analysis shown to have normal diploid (2N) DNA content**



**5.2b) Gallery of Aneuploid Nuclei (on the same scale as 5.2a) later shown to have a DNA content of 3.6N**



**5.2c) Gallery of Tetraploid Nuclei (on the same scale as 5.2a) later shown to have twice the normal DNA content**



The picture or 'digital image' of each nucleus has the relative optical density of each pixel within it stored as well as the size of each nucleus. These parameters allow for the integrated optical density (IOD) of each nucleus to be calculated a measure of the total optical density across the whole nucleus. Additionally, detailed information is recorded regarding the size, shape and variance of optical intensity across each nucleus within a population of cells.

Image cytometry has two potential advantages over flow cytometry:

- a. A smaller number of nuclei are required for accurate analysis of DNA content (Fang et al, 2004; Sauter et al, 2004; Sudbo et al, 2004) although no formal studies to quantify any loss of accuracy have been performed.
- b. The nuclei collected are viewed prior to analysis allowing lysed, cut and clumped nuclei to be excluded. This minimised the error of near diploid aneuploidy due to cut nuclei and reduced the error in the 4N fraction due to clumped nuclei.

This manual analysis of the nuclei prior to result interpretation is a potential strength of image cytometry allowing clumped or cut nuclei to be excluded from the analysis. These clumped or incomplete nuclei are responsible for many errors in flow cytometry. One criticism and potential source of bias is that this process allows human error into the analysis although if performed prior to histogram generation this bias should have been minimised. Examples of exclusions on the basis of being cut or multiple nuclei are shown in figure 5.2 above.

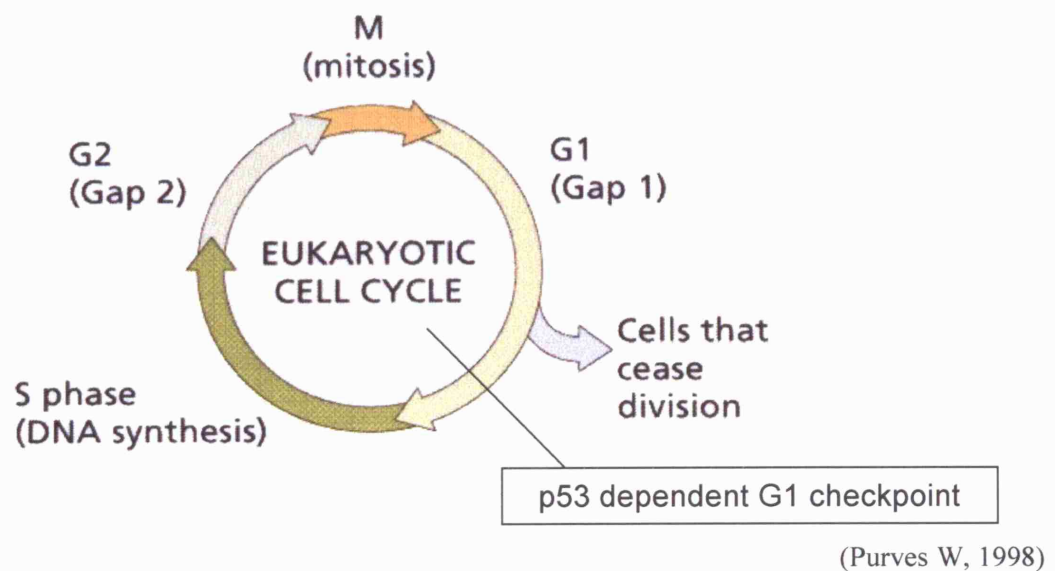
Formal correlations of flow and image cytometric findings have not been previously undertaken in Barrett's oesophagus. There have been studies, however, in other diseases that have compared these methods. These studies examined ovarian (Fleazar et al, 2003) and breast tumours (Alanen et al, 1998) and have not showed any significant differences between the methods for the detection rate of aneuploidy.

### ***Interpretation of the results of Image and Flow Cytometry***

In order to understand these results a brief and rudimentary knowledge of the normal cell cycle is necessary. Most cells are resting in phase G0 of the cell cycle. Some cells are in G1 or the growth phase and are preparing to enter DNA synthesis (or S phase) where the DNA is copied in its entirety ready for cell division. In order to enter S Phase there is a p53 dependent checkpoint at which stage cells with DNA damage are either repaired or undergo apoptosis. The inactivation of

this checkpoint is consequently an important step to aneuploidy development and carcinogenesis. After entering S phase the DNA is copied (DNA replication) and the nucleus of the cell during this stage has anywhere between two (the normal chromosomal load) and four copies of each chromosome. Following DNA replication the cell enters G2, essentially a second gap phase preparing for mitosis. Mitosis itself occurs in the M phase resulting in the formation of two identical daughter cells. In each type of cell population there is an upper limit of normal for the maximum percentage of cells that should be undergoing cell division (also known as the 4N fraction or the percent of cells with tetraploidy).

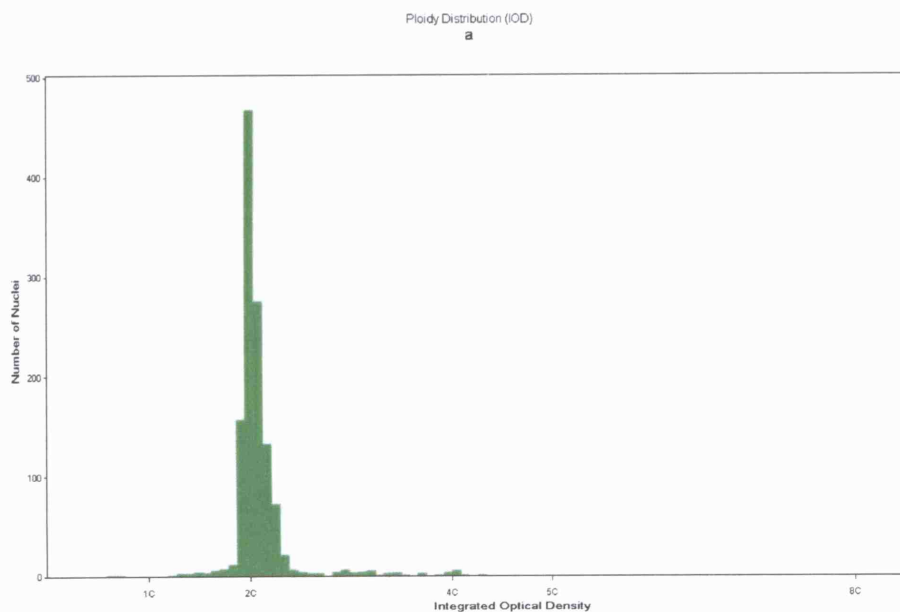
**Figure 5.3: The Cell Cycle**





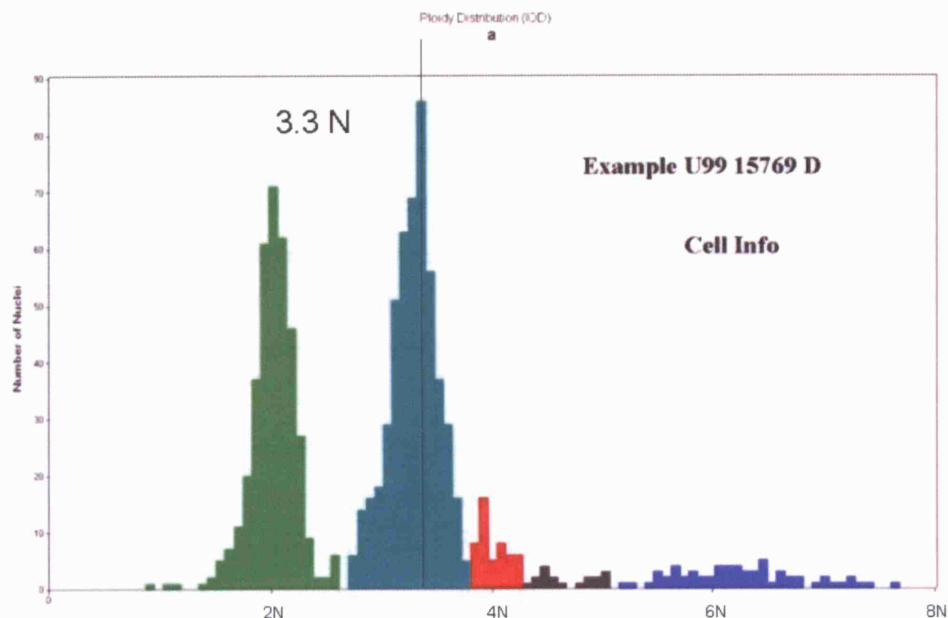
In image cytometry, a bar chart is then plotted with integrated optical density on the x axis and the frequency of nuclei on the y axis. This bar chart is called a 'histogram'. In order to calculate the relative DNA content of each nucleus the position of 2N (diploid nuclear content) is defined for each histogram separately. In line with the consensus guidelines for DNA Image Cytometry published by the European Society for Analytical Cellular Pathology, the diploid (2N) position was defined by the mode of the peak adjacent to the internal lymphocyte controls which was placed manually on the histogram (Bocking et al, 1995). The positions of all other DNA contents are then automatically placed on the histogram relative to this position.

**Figure 5.4: Example of a Diploid histogram**



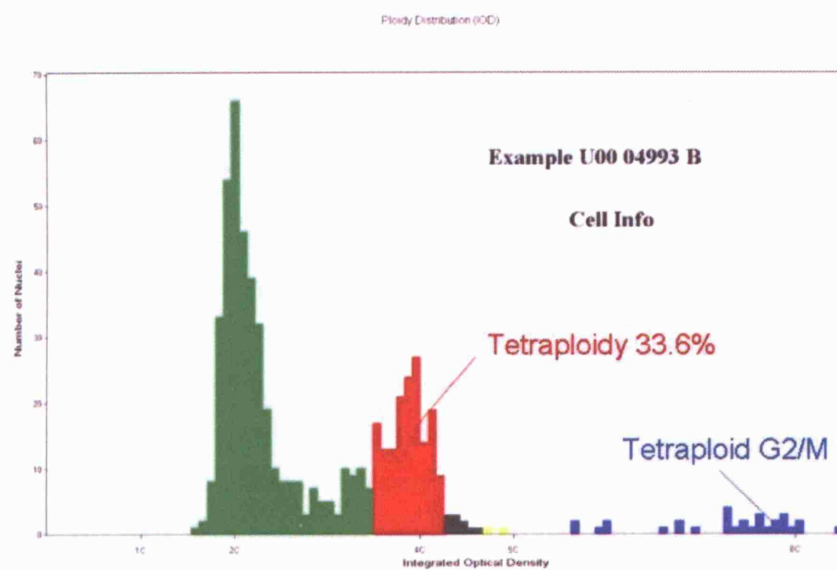
Single large peak of diploid nuclei around 2N with a small proportion of nuclei in the S Phase between 2N and 4N and a few nuclei in G2/M phase situated at 4N.

**Figure 5.5: Example of an Aneuploid histogram**



The green peak represented diploid nuclei at 2N. The turquoise peak represented aneuploid nuclei at 3.3N. The red peak was the 4N fraction and the dark blue represented the dividing aneuploid nuclei in G2 or mitosis phase of the cell cycle. The same features are represented below for a tetraploid histogram with 33.6% of nuclei in the 4N fraction.

**Figure 5.6: Example of a Tetraploid histogram**



## **5.2 Aims of this chapter**

1. Develop methodology for processing nuclei from paraffin embedded tissue for analysis using Image Cytometry (Section 5.3),
2. Validation of Image cytometry for the measurement of aneuploidy:
  - a. To assess if image cytometry is correlated with histology (5.4.1),
  - b. To assess if image cytometry is correlated with flow cytometry (5.4.2),
  - c. To measure the accuracy of image cytometry and errors introduced by collecting different numbers of nuclei for analysis (5.4.3),
  - d. To estimate the errors introduced by performing image analysis on a 40 micrometre section cut from a paraffin embedded biopsy as opposed to whole biopsy specimens being processed (5.4.4),
3. To examine whether the presence of aneuploidy before and after treatment with PDT predicts the success of therapy (section 5.5).

## **5.3 Methodology for Image Cytometry (ICM) Specimen Preparation**

### ***Paraffin Embedded Biopsies***

#### **Introduction**

Many factors are involved in liberation of nuclei including type of digesting enzyme (eg protease or pepsin), concentration of that enzyme, time of tissue exposure and the temperature of the reaction. Finally, the thickness of the cut section used has varied between 30-60µm and therefore each laboratory uses different concentrations of protease or pepsin for different lengths of time. Many papers do not report all of these variables.

With regard to the thickness of the cut section used collecting up to two 60 micrometre sections from each biopsy block would in most cases exceed the residual biopsy material. The implications of this were two fold; first and most importantly there was no residual material if further histological analysis were to be required for clinical purposes and secondly if further experiments were required then the supply of tissue would have also been exhausted. Alternatively, if a 30 micrometre section had been cut then this would have increased the proportion of cut nuclei in the

specimen and introduced potential errors (section 5.4.4). A 40 micrometre section was decided upon, in the first instance, as a balance between the need for whole nuclei for analysis and preservation of archival material.

Cost of consumables is always a relevant factor and so a small amount of protease (200 micolitre at a concentration of 5mg/ml) was chosen for each specimen to start with for this experiment. The optimal condition for speed of digestion is a temperature of 37°C which left the time of digestion as the single variable to be examined in this experiment.

### Aim

To determine a reliable method of liberating nuclei from paraffin embedded tissue for image cytometry analysis.

### Methods

In order to determine the correct length of time, therefore, to digest a 40micrometre section four different times were assessed using protease XXIV (concentration 5mg/ml, Sigma-Aldrich, Dorset, UK). Three specimens were digested for each of the following times; 1, 2, 2.5, 3 hours.

### ***Methodology for Detection of Aneuploidy from paraffin embedded tissue***

The method of processing samples for image cytometry analysis from paraffin embedded tissue was adapted from the ESACP Guidelines (Bocking et al, 1995) and the methodology published by Hedley et al (Hedley et al, 1983). This methodology can be divided into three steps; the liberation of nuclei from paraffin embedded tissue, the Feulgen staining process and then histogram generation using an automated image cytometer.

#### The Liberation of Nuclei from paraffin embedded tissue:

1. A 40 micrometre section was cut from paraffin block,
2. Paraffin removed from embedded tissue with 1.5mls of xylene (VWR, Dorset, UK) for 10minutes),
3. Centrifuge for 1 minute 13,000 rpm, remove supernatant with pipette,
4. Add 100% Ethanol 1.5mls, vortex (Vortex Genie 2, Scientific Ind, New York, USA) for 5 seconds then stand in ethanol for 5 minutes,
5. Repeat centrifuge and removal of supernatant as described above,
6. Add 1.5mls of distilled water, vortex, stand for 2minutes, centrifuge and remove supernatant (settings as above),
7. Mix 1 tablet (Sigma-Aldrich, Dorset, UK) of phosphate buffer to 200mls distilled water to create phosphate buffered saline (PBS, pH 7.4, Phosphate buffer 0.01M, 0.0027M KCl, 0.137M NaCl). Add 1.5mls vortex (5s) and the stand for 2 minutes. Centrifuge and remove supernatant as above,
8. Add 200 microlitres of protease XXIV (Sigma-Aldrich, Dorset, UK ) at concentration 5mg/ml (0.05g in 10mls PBS) at temperature 37<sup>0</sup>C, for varying times (1, 2, 2.5, 3hrs) described above,
9. Add 1.4mls PBS chilled to 4°C. Pipette entire sample and filter though 40micrometre nylon mesh cell strainer (BD Biosciences, California, USA),
10. Centrifuge for 10minutes at 1600rpm. Remove supernatant,
11. Re-suspended in PBS 200microlitres, vortex (5s),
12. Cytospin (Shandon Cytospin 4 at 1500rpm for 5minutes) using a Shandon single use cytofunnel (Thermo Scientific, Basingstoke, UK) onto Superfrost Plus (blue) microscope slide (electrostatically permanently positive charge, VWR, Dorset, UK),
13. Air dry at room temperature for 1 hour in slide holder for 12 or 24 slides.

#### Feulgen-Schiff Staining Process:

14. Place slide holder in 200mls of 4% Formalin to fix overnight,
15. Wash slide in slide holder with 200mls of distilled water in large glass trough,
16. Transfer slide holder into glass trough containing 200mls HCL 5mol/litre for 1 hour,

17. Rinse in distilled water (200mls in glass trough) for 2 minutes by transferring slide holder,
18. Place 200mls Feulgen stain (Schiff's reagent, Raymond A Lamb, Sussex, UK) in a glass trough and transfer slide holder. Leave for 2 hours in the dark at room temperature,
19. Rinse in cool running tap water 5 minutes,
20. Place slide holder into 200mls of graduated ethanol (50%, 75%, and 100%) in glass troughs for 2 minutes each,
21. Place slide holder in 200mls xylene for 2 minutes, repeat,
22. Mount with DPX mounting medium (Lamb, Sussex, UK) and add microscope glass cover slip (24x32mm, VWR, Dorset, UK).

The image cytometry in all of these experiments was performed using an automated analyser (Fairfield Imaging Ltd, Kent, UK). Histograms were generated using the methods described above:

23. The nuclei were automatically captured from the slide as described above with the maximum number of nuclei to be collected set at 2000. This number was selected due to the processing speed of image cytometer becomes significantly slower if more nuclei were collected from any individual slide,
24. This gallery of nuclei was viewed with cut nuclei and clumped nuclei excluded,
25. The histogram then calculated for IOD and the position of 2N defined,
26. The histogram was saved in an anonymised way for later interpretation using the criteria defined above,
27. Stained slides can be stored at 4°C in the dark for up to one year.

**G**

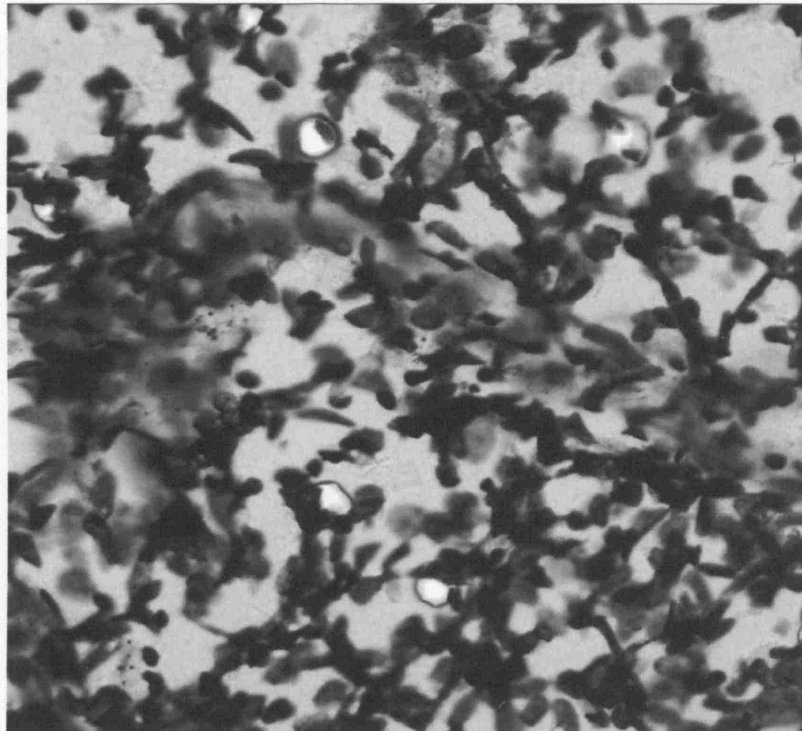
## Results

The optimal length of digestion using the above parameters for a 40µm section was 2.5 hours although 2 hours also produced workable histograms on 2/3 occasions. Overdigestion resulted in nuclear lysis or complete sample digestion and shorter times of digestion lead to the nuclei either remaining clumped or with cytoplasm attached.

**Table 5.1: Protease digestion times for paraffin embedded tissue**

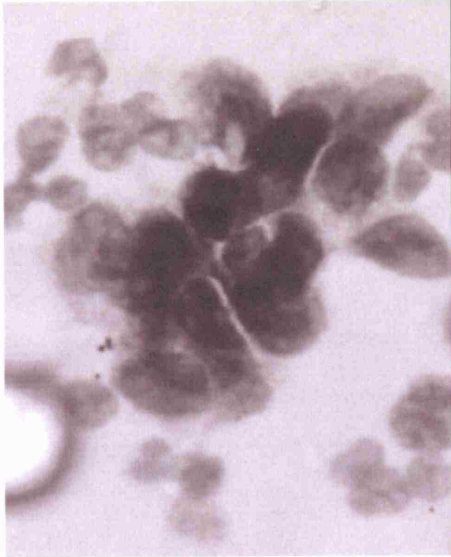
Length of Digestion	Number producing histogram	Principle problems
1 hour	0/3	Clumped/Undigested cells
2 hours	2/3	Some attached cytoplasm
2.5 hours	3/3	Good cell preparation
3 hours	0/3	Overdigested/ Absent nuclei

**Figure 5.7: Clumped and Undigested cells**

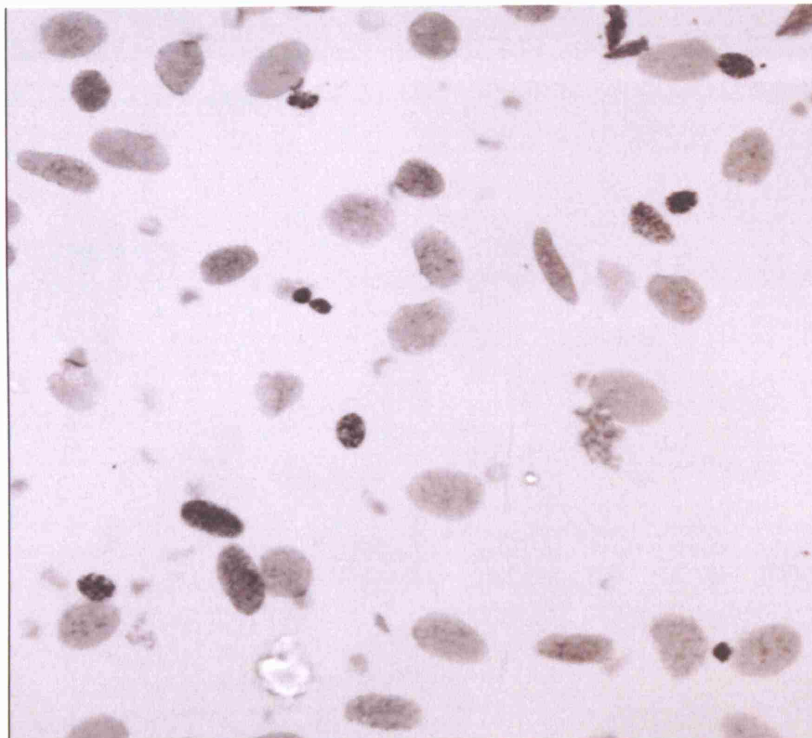




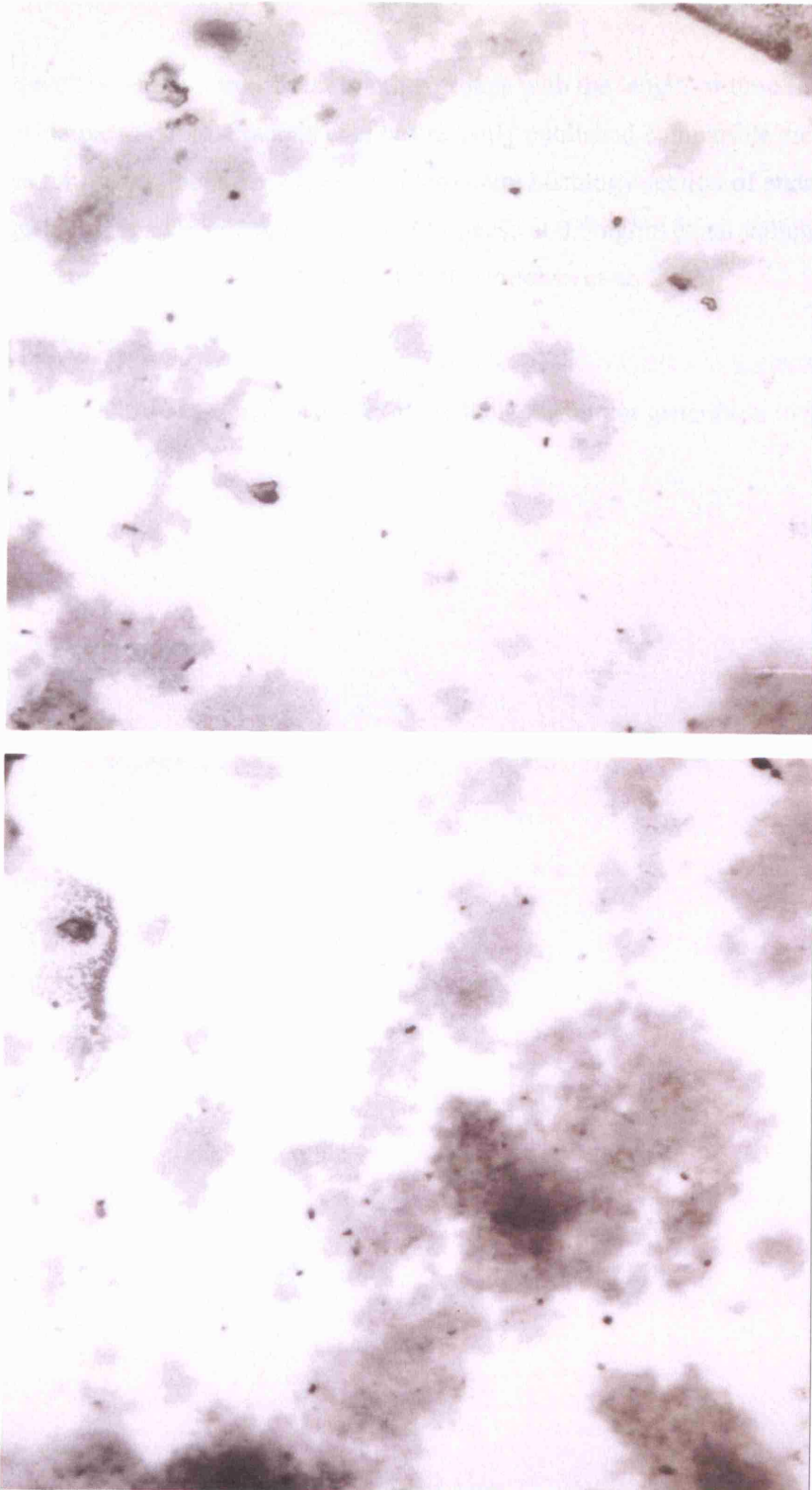
**Figure 5.8: Clumped Nuclei with cytoplasm attached**



**Figure 5.9: Good nuclei preparation, high numbers of distinct nuclei in a monolayer**



**Figure 5.10: Overdigested nuclei**



## Conclusion

The methods developed above are similar to other groups with the length of time for nuclei digestion the principle variable. Pradhan et al has recently published comparable methodology. The parameters for nuclei liberation were a 50 micrometre histology section of endometrial carcinoma digested with a lower concentration of protease at 0.5mg/ml (total volume not stated) but for a shorter length of time (1 hour) again at 37°C (Pradhan et al, 2006).

These methods have allowed for reproducible and reliable histogram generation in the studies described below.

## 5.4 Validation of Image Cytometry in the Measurement of Aneuploidy

### 5.4.1 To assess if image cytometry is correlated with dysplasia

#### Introduction and Aim:

Although aneuploidy is an independent marker of future cancer risk other groups have shown that aneuploidy is correlated with the degree of dysplasia found in Barrett's Oesophagus. Correlation of a new biomarker with those that already exists (ie histology) could be considered Phase 2 of biomarker development as described in chapter 1. The aim of this section was to demonstrate that aneuploidy measured using ICM was correlated with histology.

#### Methods:

100 samples with known histology were tested for aneuploidy and tetraploidy. 10 patients with cancer and 30 of each HGD, LGD and no dysplasia were processed as described above.

#### Results:

100 samples were processed for aneuploidy. Two patients' samples did not produce readable histograms. Of the 98 remaining samples the following results were seen.

**Table 5.2: Image Cytometry results compared to histology**

Group	n	Ca	HGD	LGD	Non-dysplastic BO
Reid et al 2000, FCM	322	>95%	66.7%	13%	
Fang et al 2004, ICM	56	100%	73%	60%	13%
Robaszkiewicz et al, 1991, FCM	96	78%	44%	11%	0%
Fennerty et al 1989, FCM	86	NA	65%	25%	8%
<b>UCLH, ICM</b>	<b>98</b>	<b>90% (9/10)</b>	<b>62% (18/29)</b>	<b>38% (11/29)</b>	<b>13% (4/30)</b>

FCM= Flow Cytometry, ICM=Image Cytometry NA=Not Assessed

(Fang et al, 2004; Fennerty et al, 1989; Reid et al, 2000; Robaszkiewicz et al, 1991)

#### Discussion:

This first step of validating aneuploidy using ICM suggests that aneuploidy correlates with histology in similar proportions to other published studies. This brief study could be considered

phase 2 of biomarker development for aneuploidy measured using ICM (Pepe et al, 2001). This study essentially repeats the work of the Seattle group but it used image as opposed to flow cytometry. The 'failure rate' to produce a working histogram using the methodology described above was 2%.

#### ***5.4.2 To assess if image cytometry is correlated with flow cytometry results from Seattle***

##### **Introduction and Aim:**

Flow cytometry, despite its difficulties, is the current gold standard for the detection of aneuploidy. The Seattle group is the world leader in its diagnosis and has published widely on the future cancer risk associated with aneuploidy in Barrett's Oesophagus. As explained earlier, image cytometry only has one case control study of future cancer risk to support its use and there have been no formal comparisons of image versus flow cytometry for the detection of aneuploidy in Barrett's Oesophagus.

The Seattle definitions of aneuploidy and tetraploidy presented in section 5.1 were used for this study. In particular greater than or equal to 6% of nuclei at 4N (3.85-4.1N) was used to define the tetraploid fraction.

The aim of this work was to determine whether image cytometry when compared to flow cytometry performed in Seattle detected aneuploidy in the same samples, are the positions of any aneuploid peaks the same and are the proportions of abnormal nuclei similar.

##### **Methods**

10 biopsy samples collected in Seattle from patients with Barrett's Oesophagus five known to have aneuploidy and five known to be diploid were studied. These samples were frozen following endoscopy, their nuclei liberated in published way by the Seattle group (Reid et al, 2000) and prior to staining for flow cytometry the sample split into two sections. The samples were then assessed in a double blind way. One was assessed using flow cytometry in Seattle, the other frozen and sent to UCH in London and processed in the normal way from step 8 above. The results from UCH, London were analysed and agreed by two independent observers (Professor M Novelli and Dr G Mackenzie) for abnormalities, the position of any abnormal peaks and the

proportion of nuclei in these peaks noted. These results along with the histograms were sent to Seattle for unblinding, comparison and further comments.

The definitions for aneuploidy and tetraploidy used are defined in the introduction above.

## Results

Both Image and Flow cytometry agreed in all five aneuploidy or tetraploidy cases as well as all five diploid cases. The table below shows the results in more detail displaying the mode and percentage of nuclei reported blind in the aneuploid and/or tetraploid peaks of each case.

**Table 5.3: Results of Image and Flow Cytometry on 10 Double-Blind analysed samples**

Case	Image Cytometry, London		Flow Cytometry, Seattle	
	Ploidy Status	Abnormal Peak (Mode, % of nuclei)	Ploidy Status	Abnormal Peak (Mode, % of nuclei)
1	Tetraploidy	Tetraploidy 11.5%	Tetraploidy	Tetraploidy 15.6%
2	Diploid	NA	Diploid	NA
3	Diploid	NA	Diploid	NA
4	Aneuploidy/ Tetraploidy	Aneuploidy (3.8N, 22%) Tetraploidy (8.1%)	Aneuploidy	Aneuploidy (3.87N 20.3%)
5	Aneuploidy	Aneuploidy (3.6N, 35%)	Aneuploidy	Aneuploidy (3.74N, 36%)
6	Diploid	NA	Diploid	NA
7	Aneuploidy	Aneuploidy (3.1N, 46%)	Aneuploidy/ Tetraploidy	Aneuploidy (3.05N, 49%)
8*	Aneuploidy	Aneuploidy (3.2N, 39%) *	Aneuploidy/ Tetraploidy*	Aneuploidy (3.26N, 24.7%) Tetraploidy (8.2%)
9	Diploid	NA	Diploid	NA
10	Diploid	NA	Diploid	NA

\* Comment from Seattle: “Case 8: The flow cytometric aneuploid 3.26N of 24.7% together with the diploid 4N of 8.5% is 33.2% which is roughly equivalent to the image cytometry 39% aneuploid with a wide CV [coefficient of variation]”.

## Discussion

This is a small study of image versus flow cytometry. Aneuploidy detection is closely correlated between image and flow cytometry with similar proportions of abnormal nuclei and the mode of the aneuploid peaks.

The measurement of tetraploidy appears slightly more prone to error. The feedback from the Seattle group suggests that the 95% confidence interval (CI) around a 4N fraction of 6% can be estimated depending on the number of nuclei analysed in the sample. Using ICM based on an average of 1000 nuclei collected the 95% CI is 4.5-7.4%. The 95% CI of flow cytometry based on collection of 20,000 nuclei per histogram is 5.5-6.5%.

Understanding this limitation of a slightly wider 95% confidence interval, a reasonable estimation of the 4N fraction can be made using image cytometry. In patients with flow cytometric abnormalities but no HGD 40% have tetraploidy as the sole abnormality (Reid et al, 2001). Furthermore, of those patients with tetraploidy 80% (39/52) have 4N fractions between 6-15% (Rabinovitch et al, 2001). Only a few patients will therefore have a true 4N fraction <7.4% and be misclassified into low risk. The vast majority would either be an overestimate or only a small underestimate still resulting in the correct overall classification with a 4N fraction >6% or tetraploidy.

### ***5.4.3 Analysis of the number of nuclei required for accurate histogram interpretation***

#### **Introduction and Aim:**

It has been suggested that only 300 nuclei or fewer are required to form an accurate histogram for the detection of aneuploidy using ICM (Sauter et al, 2004). There have not been any formal studies to date however to show if a smaller proportion of the sample is assessed whether this introduces significant errors into the detection of aneuploidy or tetraploidy. The study with Seattle above suggests that this is the case particularly for tetraploidy but other groups have successfully collected small numbers of nuclei (less than 75) and still made an assessment of aneuploidy albeit not tetraploidy (Sauter et al, 2004). The aim of this study was to assess if the histogram changes significantly when fewer nuclei are collected.

#### **Methods:**

Twenty eight biopsies, 11 with ICM abnormalities (7 with aneuploidy and 4 with tetraploidy) and 17 diploid, were systematically re-analysed using image cytometry collecting different numbers of nuclei. The gold standard was considered a histogram calculated from at least 2000 nuclei. The automated image capture was set to obtain 75, 125, 175, 225, 300, 600, 1100 and over 2000 nuclei. Histograms were then calculated and compared to the gold standard measures for overall diagnosis by blinded visual interpretation for the presence or absence of aneuploidy and tetraploidy.

Additional analysis was performed to quantify these errors using other published measures of ploidy status using image cytometry (Bocking et al, 1995). These measures include the DNA Index (DI) which equates to the mean nuclear integrated optical density relative for the whole histogram divided by the diploid integrated optical density, the proportion of cells greater than 2.5N (fraction >2.5N) and the 4N fraction. The accuracy of each of these measures (DI, >2.5N and 4N fraction) for histograms created from small samples of nuclei collected from the same slide were then calculated compared to the gold standard (>2000 nuclei collected) as a standardised difference or proportional inaccuracy to the gold standard using the following formulae:

Standardised Difference in 4N fraction=  $\frac{\text{Measured 4N fraction in histogram of } n \text{ collected nuclei}}{\text{Measured 4N fraction in gold standard histogram}}$



$$\text{Standardised Difference } >2.5N = \frac{\text{Measured fraction of nuclei } >2.5N \text{ in histogram}}{\text{Measured fraction of nuclei } >2.5N \text{ in gold standard histogram}}$$

$$\text{Standardised Difference DI} = \frac{\text{Measured DI in histogram of } n \text{ collected nuclei}}{\text{Measured DI in gold standard histogram}}$$

For each histogram analysing a different number of collected nuclei the inaccuracies compared to the gold standard of >2000 nuclei would be plotted. The number of nuclei collected on the x axis and the standardised difference of the 4N fraction, the fraction of nuclei >2.5N and DI on the y axis. A quadratic regression was then performed and a line of best fit added to the graph with 95% CI then calculated and plotted to estimate errors.

#### Results:

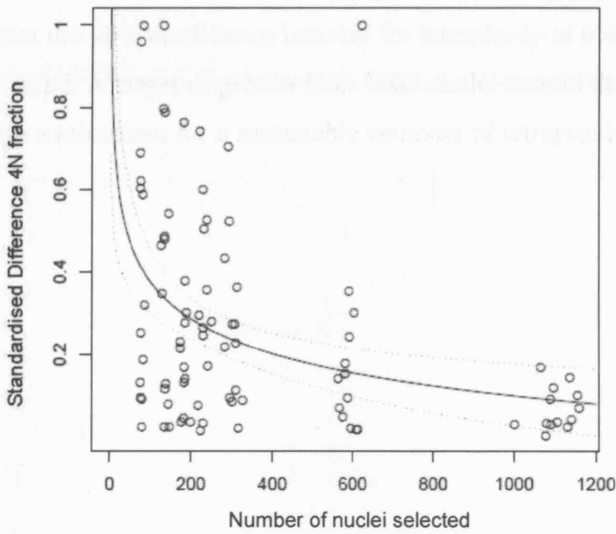
Aneuploidy appeared to be detectable in a reliable way to the lower limit of this experiment (75 nuclei collected). The detection of tetraploidy was more problematic with only 2/4 tetraploid histograms correctly identified if less than 600 nuclei were collected and if less than 300 nuclei were collected 1 out of 17 diploid histograms was misclassified as tetraploid. (Table 5.4)

This experiment appears to confirm the results of the small comparison of image and flow cytometry above. Even when collecting small number of nuclei from a sample an aneuploid fraction was still detected and in figure 5.13 the estimated errors of doing this were small. For example at 200 nuclei there was less than a 5% change in the DNA Index of the histogram. The difficulty in collecting less than 600 nuclei in this series for the diagnosis of tetraploidy is highlighted again in figure 5.12. From this small sample if only 600 nuclei were collected then the change from the estimated 4N fraction in the gold standard histogram of >2000 nuclei was as much as 10%. Assuming 6% is the ideal cut off for future cancer prediction then the estimated sampling error from just collecting fewer nuclei compared to a histogram of 2000 nuclei is 4.8-7.2%. These results confirm the reduced precision in the 4N fraction as few nuclei are collected as suggest by the Seattle group and explain the reason that 2 out of the 4 tetraploid cases would be missed on histogram interpretation if <600 nuclei were collected.

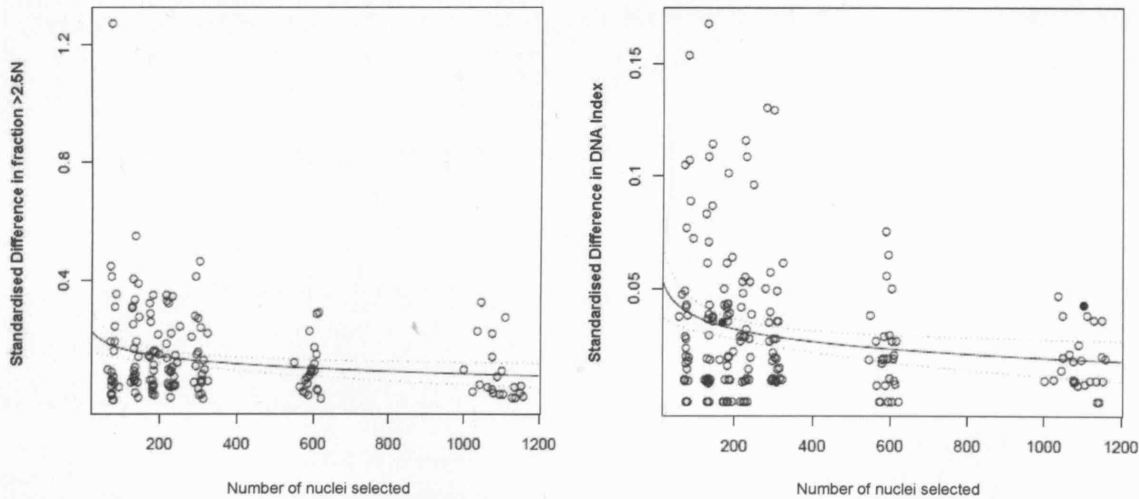
**Table 5.4: Blinded single observer interpretation compared to gold standard depending on number of nuclei per slide**

	75	125	175	225	300	600	1100	>2000 Gold Standard
Aneuploid	7/7	7/7	7/7	7/7	7/7	7/7	7/7	7
Tetraploid	2/4	2/4	2/4	2/4	2/4	4/4	4/4	4
Diploid	16/17	17/17	16/17	16/17	17/17	17/17	17/17	17

**Figure 5.11: Quantitative measures of Inaccuracies in collecting fewer nuclei. The proportional error in 4N fraction**



**Figure 5.12: Quantitative Measures of Proportional error in average DI and Fraction of nuclei >2.5N**



Comparison to defined ‘Gold Standard Histogram’ of >2000 nuclei collected from the same sample

## Discussion:

Aneuploidy was detectable even when as few as 75 nuclei were collected. This is in agreement with one image cytometry paper examining the presence of aneuploidy from breast cytology but this group made no attempt to diagnose tetraploidy (Sauter et al, 2004). For the diagnosis of tetraploidy it would appear that at least 600 nuclei are required but the greater the number of nuclei analysed from the slide the lower the 'sampling error'. This result confirms the observation from Seattle stating that the 95% confidence interval for tetraploidy at 6% is wider the smaller the number of nuclei collected. A target of greater than 1000 nuclei should therefore be set for all other experiments with a minimum for a reasonable estimate of tetraploidy being 600 nuclei.

#### ***5.4.4 Errors introduced by cutting sections from biopsies***

##### **Introduction and Aim:**

Biopsies for analysis can be processed either from 'fresh' un-embedded biopsies or from paraffin embedded tissue and a cut section. The biggest potential problem in cutting a section from a paraffin block is that a proportion of the nuclei are cut and are consequently removed from the analysis. Aneuploid nuclei are larger than diploid nuclei and a greater proportion of these are, therefore, destroyed prior to analysis. In the use of historical tissue or the processing of tissue from other hospitals the processing of paraffin embedded samples is necessary for practical reasons. The aim of this section is to estimate the size of this 'cutting' error and how it might be corrected for.

In the methodology described above a 40 micrometre section is cut from a paraffin block and a proportion of nuclei are cut at the edges of this section excluding them from the analysis. Were all the nuclei that are cut excluded from the analysis and were aneuploid/tetraploid nuclei cut at the same rate as diploid nuclei then no error occurs in the fraction of nuclei in any part of the histogram and there would be no subsequent change in the aneuploid or tetraploid fractions. However, aneuploid and tetraploid nuclei may be significantly larger than diploid nuclei and hence a potential source of error with subsequent underestimation of these fractions.

It has been suggested that nuclear size increases with the grade of dysplasia (Polkowski et al, 1998) and because dysplasia is correlated with the presence of aneuploidy and tetraploidy it is entirely plausible that these nuclei are not only denser but also larger than diploid nuclei within a sample. If they are larger then these nuclei are likely to be cut with a greater frequency than the smaller diploid nuclei resulting in a relative underestimate of both the 4N and aneuploid fractions. This is likely to be far more significant with the diagnosis of tetraploidy as the cut-off between low and high risk is quantitative and set at a value of only 6% (Vaughan et al, 2005). The definition of aneuploidy also includes a fraction (second 'aneuploid' peak greater than 2.5% of the population) but the vast majority of these samples have far larger aneuploid fractions with few approaching this level. In one of our studies the proportion of aneuploid histograms with an aneuploid fraction of 2.3-5% was approximately 5% (3/64 aneuploid histograms).

## Methods:

Two assumptions were made for this analysis. The first was that although the nuclei are not spherical they can be treated as such provided there is no systematic difference in the eccentricity of the nuclei with aneuploidy. Eccentricity can be defined as the longest length divided by the shortest length of each nucleus. This eccentricity (also known as form factor) was also measured by the image cytometry machine. This assumption was tested prior to presentation of the results below. The second assumption was that all the cut nuclei within the study population are removed. As stated, one advantage of image cytometry is the ability to 'view' nuclei prior to analysis. This results in the removal of 'cut' nuclei preventing the inclusion and subsequent error of including incomplete nuclei in the analysis.

### **Estimate of proportion cut is dependent on the radius of the nuclei:**

Data from galleries relating to the average size of nuclei in the diploid peak and those in the aneuploid and tetraploid peaks were acquired from the image cytometer. These data were extracted and analysed. The proportion of nuclei cut by sectioning at 40 micrometres was then estimated and the effect of this on the tetraploid and aneuploid fractions can then be calculated. The following formulae were calculated for this estimation.

The population of nuclei under study can be considered as the nuclei whose centre falls within the 40 micrometre section. The proportion of nuclei cut can be estimated as the proportion of the nuclei whose centre lands within a radius width of the edge of the section multiplied by 2 (two edges to the section). See figures 5.14 and 5.15. This is reliant on the assumption that the nuclei are distributed randomly in the histological section. This can therefore be expressed as the formula:

$$\text{Estimate of Proportion of Diploid Nuclei Cut} = 2 * \left( \frac{Rd}{M} \right)$$

Where,  $Rd$ = average radius diploid nuclei and  $M$ = histological section width (micrometre).

**Figure 5.13: Schematic of 40µm section**

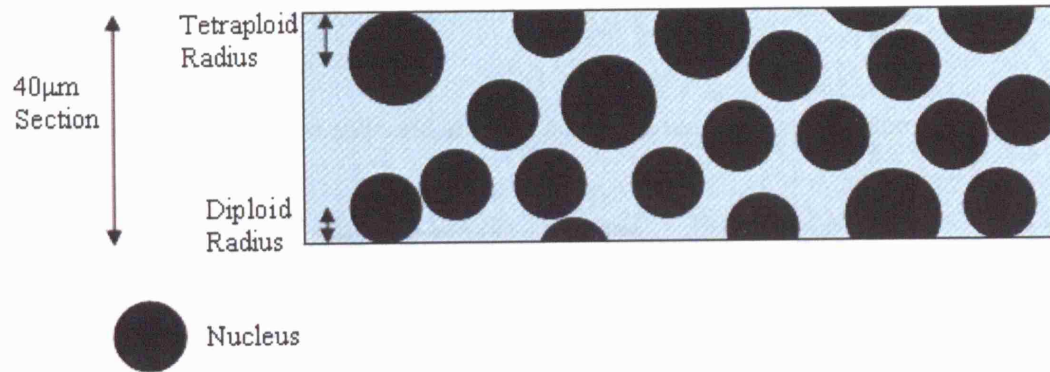
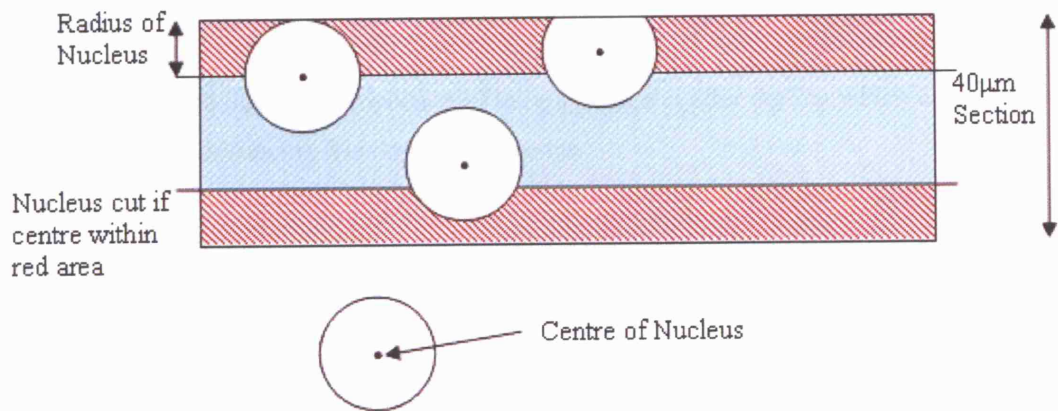


Figure 5.14 above shows that a proportion of the nuclei in a section are cut. The larger the nuclei the large the proportion of nuclei cut and subsequently excluded compared to the smaller nuclei. Figure 5.15 below shows the positions where a nucleus is cut if its centre lies within that nucleus's radius of the edge of the section.

**Figure 5.14: Schematic of positions where nuclei are cut in the 40µm section**



$$\text{Therefore, the estimate of the Proportion of Tetraploidy Nuclei Cut} = 2 * \left( \frac{Rt}{M} \right)$$

Where,  $Rt$  = average radius tetraploid nuclei and  $M$  = histological section width (micrometre).

The effect of these different proportions of cut nuclei can then be estimated using the following formulae. The value of tetraploid felt to be significant is 6% or 0.06. This can be represented as:

$$\frac{Nt}{Nt + Nd} = 0.06 \quad \text{transforms by inversion to} \quad 1 + \frac{Nd}{Nt} = \frac{1}{0.06}$$

where,  $N_t$ = True Number of Tetraploid Nuclei,  $N_d$ = True number of diploid nuclei

This can be further rearranged to show: (i)  $\frac{N_d}{N_t} = \frac{1}{0.06} - 1 = 15.667$

Observed number of nuclei collected after cut nuclei are removed can be expressed as:

$$(ii) Ot = N_t * St \quad \text{and} \quad (iii) Od = N_d * Sd$$

where,  $O_t$ =Observed tetraploid,  $O_d$ = Observed diploid after cut nuclei removed and  
 $S_t$ =Proportion of tetraploid nuclei successful collected (uncut),  $S_d$ =Proportion of diploid nuclei  
 successfully collected

We want to find the ratio of diploid to tetraploid nuclei actually collected (observed ratio),  $O_d/O_t$ .  
 Substituting (ii) and (iii) gives:

$$(iv) \frac{Od}{Ot} = \frac{(N_d * S_d)}{(N_t * S_t)} = \left( \frac{N_d}{N_t} \right) * \left( \frac{S_d}{S_t} \right) = 15.667 * \left( \frac{S_d}{S_t} \right)$$

The goal is to find the observed proportion of tetraploid nuclei after cutting which corresponds to 6% find fresh biopsies (uncut). This can be defined as:

$$(v) \frac{Ot}{Ot + Od} = 1 / \left( \frac{Od + Ot}{Ot} \right) = 1 / \left( 1 + \frac{Od}{Ot} \right)$$

Substituting equation (iv) into (v) gives:

$$\text{Observed proportion of tetraploid nuclei} = 1 / \left( 1 + 15.667 * \left[ \frac{S_d}{S_t} \right] \right)$$

An equivalent analysis for aneuploid nuclei can be carried out substituting 0.06 in formula (i) for 0.025 which results in the formula:

$$\text{Observed proportion of aneuploid nuclei} = 1 / \left( 1 + 39 * \left[ \frac{S_d}{S_a} \right] \right)$$

where  $S_a$ = Proportion of aneuploid nuclei observed after cutting

## Results:

Ten biopsies with non-dysplastic/LGD in Barrett's Oesophagus, 10 with HGD and 5 with intramucosal cancer were scanned using ICM resulting in 37,457 nuclei having their parameters assessed.

With respect to the first assumption above there was no difference in the eccentricity of the nuclei between aneuploid/tetraploid nuclei and diploid nuclei. The average form factor (longest diameter divided by short diameter) from the diploid nuclei was 1.52 and for aneuploid or tetraploid nuclei 1.54. The t-test reported a non-statistically significant p value of 0.60.

The average diameter of diploid, aneuploid and tetraploidy nuclei were as follows.

**Table 5.5: Average size of diploid, aneuploid and tetraploid nuclei in micrometres**

ICM Result	Number of nuclei	Average Diameter (µm)	SD	% Cut (40µm section)	Estimated Percent of total nuclei uncut
Diploid	32195	9.66	1.99	23.9	76.1%
Tetraploid	1296	13.06	2.09	32.7	67.3%
Aneuploid	3966	12.61	2.26	31.5	68.5%

The additional proportions cut in the aneuploidy and tetraploidy groups will affect the size of abnormalities in an abnormal histogram. These errors can be estimated depending on the cut off used to define abnormal cell populations.

If using the Seattle groups definitions of greater than 2.5% nuclei to define an aneuploidy cell population then the underestimate would be small and the new cut off taken as:

$$\text{Observed proportion of aneuploid nuclei} = 1 / \left( 1 + 39 * \left[ \frac{Sd}{Sa} \right] \right) = 1 / \left( 1 + 39 * \left[ \frac{76.1}{68.5} \right] \right) = 0.0226$$

Or 2.3%



For tetraploidy abnormal flow cytometry is greater than 6%. The corrected cut off for 6% tetraploidy using a 40micrometre section is:

$$\text{Observed tetraploidy proportion} = 1 / \left( 1 + 15.667 * \left[ \frac{Sd}{St} \right] \right) = 1 / \left( 1 + 15.667 * \left[ \frac{76.1}{67.3} \right] \right) = 0.053$$

Or 5.3%.

Discussion:

This experiment suggested that because the nuclei of aneuploid and tetraploid cell populations are larger they are cut proportionally more than diploid nuclei. This is of minor significance for aneuploidy as small aneuploid peaks between 2.3-5% are rare (less than 5% of all aneuploid histograms). Due to cutting, however, a small error did occur and could be estimated as 0.24% relating to a significant aneuploid peak greater than 2.3%.

For tetraploidy, this is potentially a far bigger problem. Forty percent of all aneuploid patients have lone tetraploidy (no HGD or aneuploidy elsewhere) as their abnormal flow cytometry predicting future cancer risk with a 4N fraction of >6% (Rabinovitch et al, 2001). It is therefore important to account for this 'cutting error'. The data above suggested that the significant percentage should be reduced from 6% for freshly prepared specimens to 5.3% for a 40 micrometre section cut from paraffin embedded tissue.

#### ***5.4.5 Summary of the Image Cytometry Technique for the detection of aneuploidy***

Image and flow cytometry appear to be closely correlated. The size and modal position of peaks of nuclei with aneuploidy appear to be very similar with both image and flow cytometry.

There are however weaknesses with any methodology. The methodology above has been refined to create a reliable histogram from a 40 micrometre cut section from a paraffin embedded biopsy collecting on average over 1100 nuclei per sample. In order to understand the errors faced by employing this methodology the experiments above have focused on the number of nuclei collected and the proportion of nuclei cut with the resultant changes in relative frequency of these nuclei and therefore the errors expected.

The data above would appear to suggest a significant error in the estimation of the 4N fraction if less than 600 nuclei are collected. If on average 1000 nuclei are used to calculate the histogram the 95% confidence interval for the detection of a 4N fraction of 6% is 4.5%-7.4%. Although 40% of patients have sole tetraploidy only 80% of these have a 4N fraction between 6-15% with the other 20% having very large tetraploid fractions >15%. Assuming even spread of 4N fractions within this range, in practice this would only result in the incorrect classification of high risk patients with tetraploidy as normal in up to an estimated 2% of cases. Collecting an average of 1000 nuclei would therefore seem reasonable with the minimum for 4N fraction estimation being 600 nuclei. The errors for cutting nuclei were estimated above and resulted in a suggested cut-off of 5.3% compared to the 6% suggested by the Seattle cohort for freshly prepared nuclei.

Although processing fresh biopsies rather than embedded samples may be preferable the errors for measuring aneuploidy from cut sections are small enough to allow a valid interpretation of the presence of aneuploidy and tetraploidy. In summary, therefore, the definitions for image abnormalities using the above methodology for future studies were defined as:

***Aneuploidy:*** a second peak of greater than 2.3% of the nuclei in a histogram not between 3.85N and 4.1N.

***Tetraploidy:*** considered present if the 4N peak (3.85-4.1N) consists of >5.3% of nuclei in a histogram of more than 600 nuclei collected.

These definitions were used in the following case control study of future cancer risk following PDT.

## **5.5 Aneuploidy following PDT and future risk of relapse to HGD or adenocarcinoma**

### *5.5.1 Introduction*

There have been many studies trying to define a patient's future cancer risk based on a histological diagnosis of dysplasia in BO. Although the results of all these studies suggest significantly increased risk of cancer with HGD the 5 year cancer rates vary widely between 13-59% (Montgomery et al, 2001; Rabinovitch et al, 2001; Schnell et al, 2001; Spechler et al, 2001). Aneuploidy has been shown to be an independent risk factor for the development of cancer in untreated BO (Rabinovitch et al, 2001; Reid et al, 2000; Teodori et al, 1998).

Until recently, oesophagectomy has been the only widely available treatment option albeit with its high mortality and serious morbidity rates in this predominantly elderly population (Dimick et al, 2003; Dimick et al, 2005; Fernandez and Meyers, B. F., 2004; Hulscher et al, 2001; Keighley, 2003; Patti et al, 1998; Sihvo et al, 2004). Furthermore, the quality of life of patients undergoing either transhiatal or transthoracic resection did not return to baseline for 12-24 months (Blazeby et al, 1995; de Boer et al, 2004). Many patients are therefore either unwilling or are considered unfit to undergo such a major procedure.

Minimally invasive approaches for disease limited to the mucosa may be better and carry fewer risks. Photodynamic therapy (PDT) is the best-studied method for mucosal ablation in BO. PDT has the advantage that light can be evenly and circumferentially distributed over the whole treatment area and is therefore particularly attractive for mucosal disease. PDT also appears to spare collagen as tissue temperature remains unchanged preserving structural integrity and reducing the risk of perforation (Barr et al, 1987). Photofrin PDT has been reported to have a success rate in the eradication rate of HGD of between 50-80% at 4 years follow up (Overholt et al, 1999; Overholt et al, 2005) and reduces future cancer risk by half to 14% (Overholt et al, 2005).

While very much safer than surgery, PDT does still have side effects. Photofrin, the only currently licensed photosensitiser for the treatment of HGD in BO, is associated with a prolonged light sensitivity of between 1-3 months and an oesophageal stricture rate of around a third (Overholt et al, 1999). The newer and potentially safer photosensitiser, ALA, does not have these

complications and appears to have good eradication rates for HGD of 75-89% (Mackenzie et al, 2007; Pech et al, 2002) although no head-to-head comparisons have been performed.

Several groups have shown that residual Barrett's mucosa following ablative therapies may harbour genetic abnormalities particularly if they were present prior to PDT. The biomarkers examined to date include p53 abnormalities, Ki67 proliferation, p16 methylation and aneuploidy. None of these studies however have compared the patients with residual abnormalities with a control group and some have not associated persistent abnormalities with later relapse to HGD or cancer (Hage et al, 2004; Hage et al, 2006; Krishnadath et al, 2000).

These residual genetic abnormalities are important because they suggest a possible reason for late relapse following PDT. Hage et al in 2006 suggested that because it is difficult to detect these abnormalities the goal of treatment should be complete Barrett's elimination (Hage et al, 2006). Complete removal of all BO is a difficult goal with any minimally invasive therapy at present and also remains a problem after oesophagectomy and one study reported a 40% Barrett's recurrence rate (16/40 patients) 36 months after surgery (Dresner et al, 2003). If a simplified method could be found to detect genetic abnormalities post therapy then early identification of these relapses may be possible.

One of the limitations of all ablative therapies is this unpredictability of which patients will relapse post treatment. It is therefore necessary to perform regular surveillance endoscopy and biopsy in all these patients. This is both unpleasant for patients and expensive. The current recommendation for Photofrin PDT is 8 endoscopies in five years follow up (Panjehpour and Overholt, B. F., 2006).

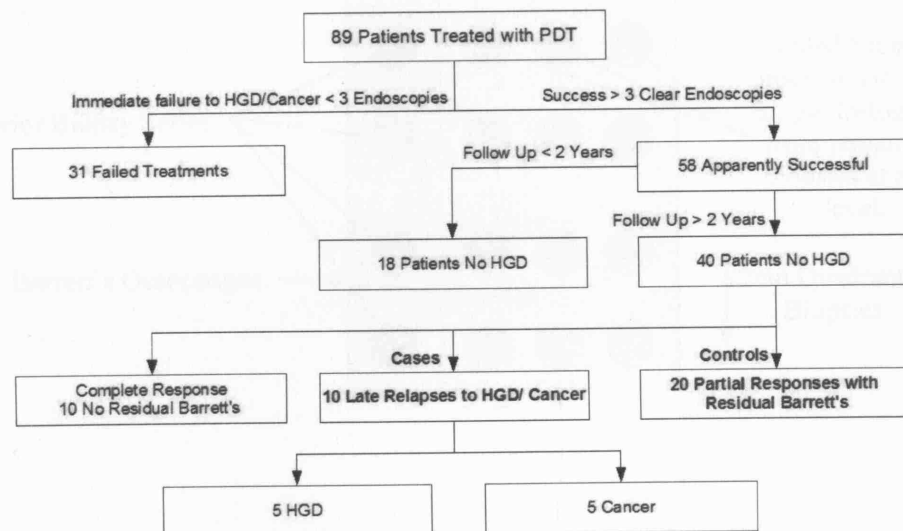
#### *5.5.2 Aims*

The two aims of this study were to assess if the presence and extent of aneuploidy prior to treatment can predict the success of therapy and whether the presence of residual aneuploidy 2-6 months after PDT predicted future relapse after apparent successful histological eradication of HGD.

### 5.5.3 Methods

**Patients:** This nested case control study occurred within a cohort of 89 patients treated with PDT for HGD or intramucosal cancer in BO. Of these patients 31 immediately failed treatment on histology (<3 clear endoscopies) and were excluded. Of the apparently successful 58 patients, 18 have had <2years follow up and were also excluded as were the 10 patients with complete squamous re-epithelialisation who have not relapsed to HGD or cancer. Of the remaining 30 patients with apparently successful eradication of HGD on histology but residual Barrett's mucosa, 10 patients have relapsed. Five patients in whom PDT initially cleared HGD later developed recurrent HGD and 5 others developed invasive cancer. The remaining 20 patients who have not developed cancer or recurrent HGD were selected as controls with follow up greater than 2 years. (Figure 5.15)

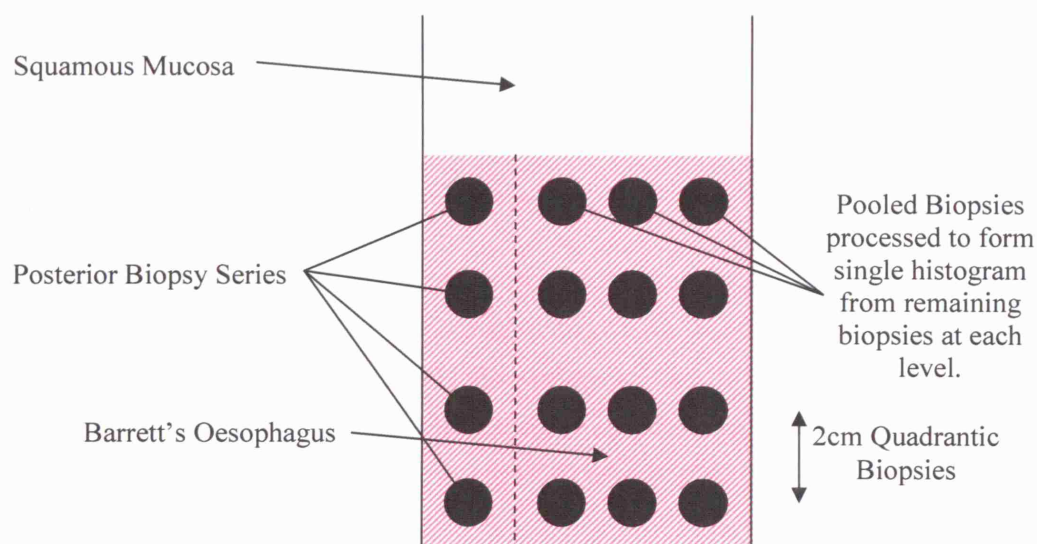
**Figure 5.15: Flow Chart of Patient Selection for Nested Case Control Study**



The 10 cases were chosen as patients who had apparently successful PDT with at least three endoscopies clear of HGD following treatment but these patients then relapsed to HGD or cancer. The controls were selected as all patients with some residual Barrett's Oesophagus and a follow up of greater than 24 months.

**Image Cytometry:** Two samples were processed for aneuploidy from each 2cm of residual Barrett's Oesophagus. One sample was from a biopsy from the posterior wall and the other sample created by pooling the three other biopsies taken at the same level. These biopsies were taken both before and 2-6 months after therapy. The Seattle group previously have used only posterior biopsies every 2cm of Barrett's oesophagus to detect aneuploidy in a so-called 'Posterior Biopsy Series' but in this study because of the potential reduction in any field effect of aneuploidy by PDT the other biopsies at each 2cm biopsy level were combined together and processed as 'Pooled Biopsies'.

**Figure 5.16: Schematic of biopsies processed for aneuploidy pre and post PDT**



The biopsies were processed using the methodology described above for histogram production using image cytometry. The definitions of image abnormalities used in this study were based on those defined by the Seattle group with the adjustments made for our methodology (chapter 5.4.5):

**Aneuploidy:** a second peak of greater than 2.3% of the nuclei in a histogram not between 3.85N and 4.1N.

**Tetraploidy:** considered present if the 4N peak (3.85-4.1N) consists of >5.3% of nuclei in a histogram of totaling more than 600 nuclei collected.

The histograms were reported blindly by two independent observers who met to agree a consensus in cases of disagreement.

The presence of aneuploidy and tetraploidy before and after treatment and the number of 2cm levels of BO with aneuploidy were documented for each patient. Additionally, the previously reported histology was recorded and these results compared between groups.

**Statistical Methods:** The differences in pre-treatment parameters (age, length of BO and levels of pre-treatment HGD) between groups were compared using t-tests and Fishers exact test. The differences between the cases and controls for presence of aneuploidy before and after treatment and the presence of LGD were analysed using Fishers Exact test.

#### *5.5.4 Results*

##### *Patients*

The 10 patients who developed cancer or recurrent HGD did so after a mean of 24 months (10-64 months). Five of the cases have developed cancer and 5 have relapsed to HGD. The 20 controls were clear of HGD or cancer and all had follow up greater than 24 months (Median 37 months, range 24-60 months). There were no statistical significant differences between cases and controls with regard to length of Barrett's and levels of HGD prior to PDT. See Table 5.6.

##### *Histogram Interpretation*

The two blinded observers reached consensus in all 30 patients' histograms. These 30 patients had a total of 363 samples processed for aneuploidy. In the 10 cases 61 samples were processed from before PDT and 58 samples post therapy. In the 20 controls 126 samples were processed prior to treatment and 118 post treatment.

##### *The presence and extent of aneuploidy prior to PDT*

There was no statistically significant difference in the presence of aneuploidy between the groups before PDT. Eight out of the ten cases (80%) and 13/20 (65%) controls had aneuploidy prior to therapy ( $p=0.67$ ). Furthermore, there were no statistical differences between the median number

of 2cm biopsy levels with aneuploidy present prior to treatment with both groups having a median of 1 and range of 1-4 levels. See Table 5.6.

*Presence of aneuploidy post PDT in the absence of recurrent HGD*

When both a posterior series and the patient's pooled biopsies were included in the analysis 9/10 cases (90%) and 2/20 controls (10%) had aneuploidy following treatment (Fisher's Exact Test,  $p < 0.0005$ , table 5.7). Of these ten cases all five of the patients that have developed cancer had residual aneuploidy demonstrated post therapy but no HGD demonstrated prior to cancer development. In the five cases of relapse to HGD four of the five (80%) had residual aneuploidy found post therapy. The odds ratio of relapsing to HGD or cancer for the presence of aneuploidy post PDT is 81 (95% CI 6.5-1017).

**Table 5.6: Pre-treatment patient parameters**

	Age †	Length of Barrett's Median, (Range) †	Level of HGD Median, (Range) †	Aneuploidy before PDT †	Levels of aneuploidy, Median (Range) †
Cases	70 (46-82)	6, (3-13)	1, (1-6 levels)	8/10	1, (1-4)
Controls	70 (56-89)	5, (1-11)	1, (1-3 levels)	13/20	1, (1-4)

† No statistically significant difference

**Table 5.7: Results of case control study displaying rates of residual aneuploidy 2-6 months post PDT**

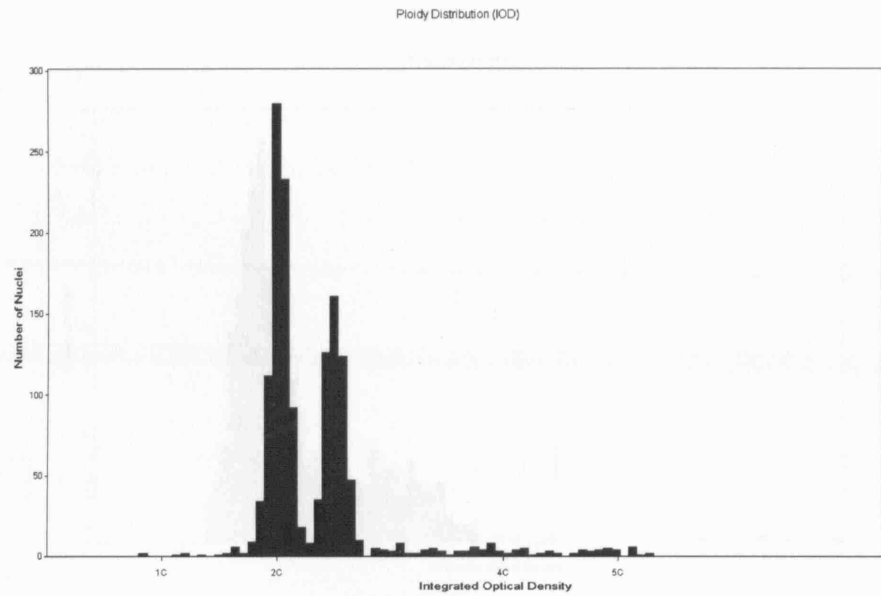
	Aneuploidy after PDT ♦	No Aneuploidy ♦	Total
Cases	9	1*	10
Controls	2	18	20
Total	11	19	30

\* Relapsed to HGD not cancer

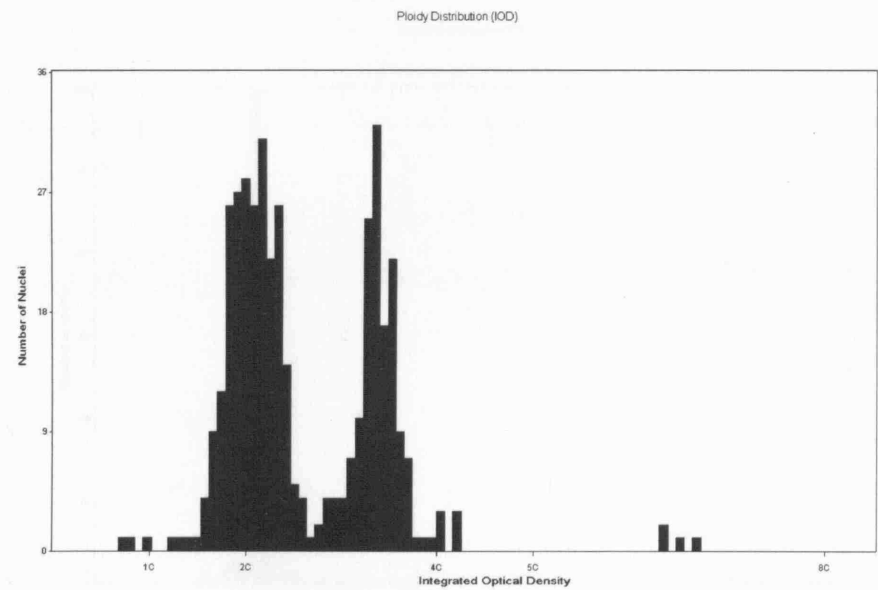
♦ Fishers Exact Test: p Value <0.0005



**Figure 5.17:** Histograms taken from a case before and after PDT

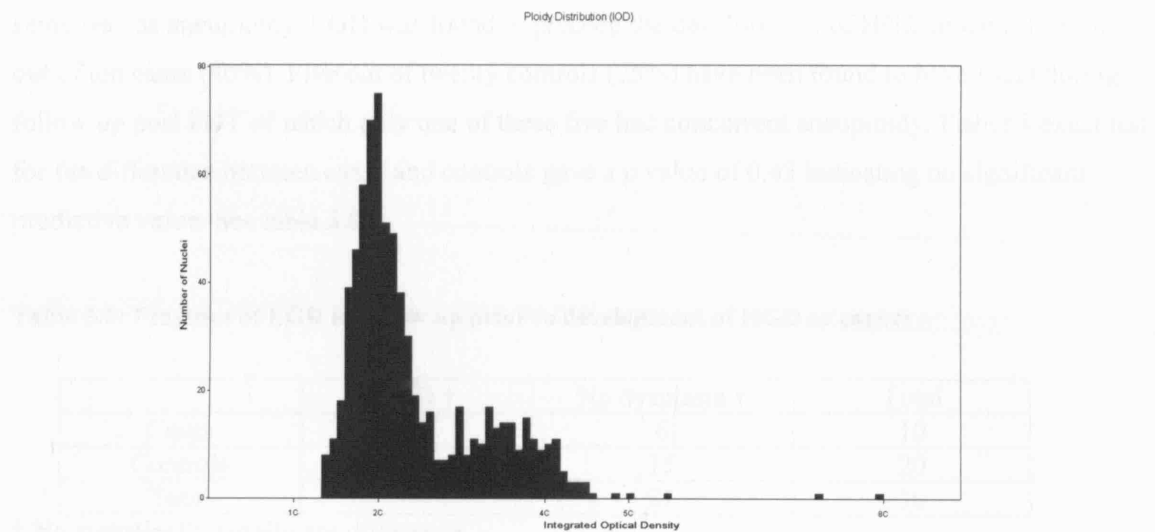


**5.17a:** Aneuploid histogram with peak at 2.6N prior to PDT

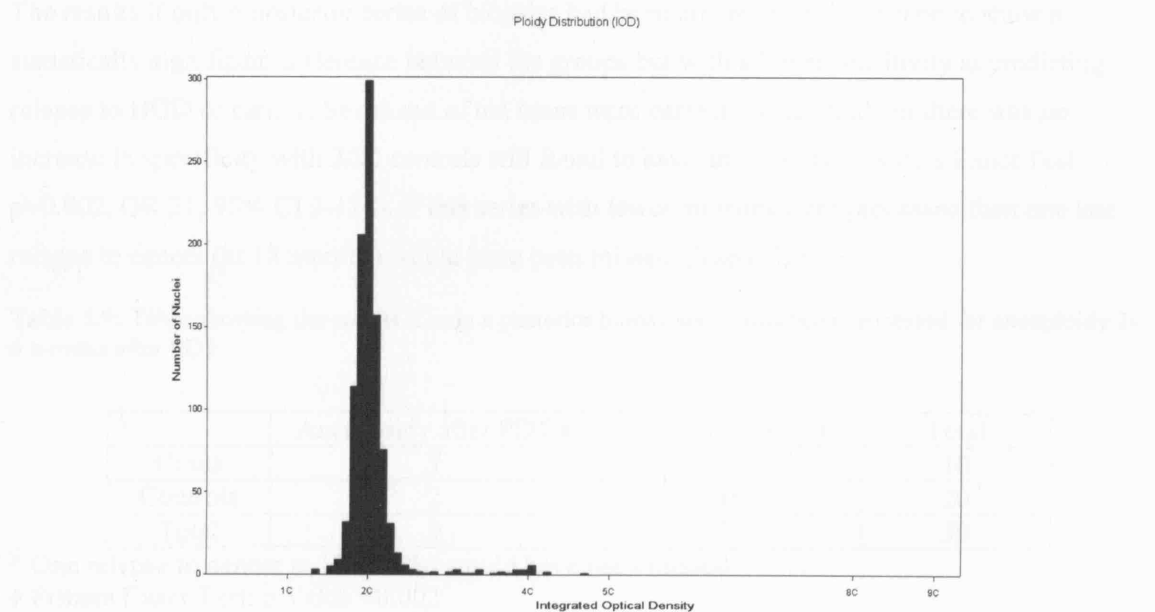


**5.17b:** Persistent Aneuploidy with peak at 3.3N post PDT

**Figure 5.18:** Histograms taken from a control before and after PDT



**5.18a:** Control with aneuploid histogram (aneuploid peak at 3.6N) prior to PDT



**5.18b:** Control with no residual aneuploidy post PDT

#### *Histology during follow up*

The presence of low grade dysplasia (LGD) after PDT did not appear to predict relapse in the same way as aneuploidy. LGD was found to precede the development of HGD or cancer in four out of ten cases (40%). Five out of twenty controls (25%) have been found to have LGD during follow up post PDT of which only one of these five had concurrent aneuploidy. Fisher's exact test for the difference between cases and controls gave a p value of 0.43 indicating no significant predictive value. See table 5.8.

**Table 5.8: Presence of LGD in follow up prior to development of HGD or cancer**

	LGD †	No dysplasia †	Total
Cases	4	6	10
Controls	5	15	20
Total	9	21	30

† No statistically significant difference

#### *Detection of aneuploidy if only a posterior biopsy series had been processed*

The results if only a posterior series of biopsies had been processed did continue to show a statistically significant difference between the groups but with a lower sensitivity at predicting relapse to HGD or cancer. Seven out of ten cases were correctly identified but there was no increase in specificity with 2/20 controls still found to have aneuploidy (Fisher's Exact Test  $p=0.002$ , OR 21, 95% CI 3-154). If this series with fewer biopsies were processed then one late relapse to cancer (at 18 months) would have been missed. (Table 5.9)

**Table 5.9:** Table showing the results if only a posterior biopsy series had been processed for aneuploidy 2-6 months after PDT

	Aneuploidy after PDT ♦	No Aneuploidy ♦	Total
Cases	7	3*	10
Controls	2	18	20
Total	9	21	30

\* One relapse to cancer at 18 months would have been missed

♦ Fishers Exact Test: p Value <0.002

### *5.5.5 Discussion*

The presence and extent of aneuploidy prior to treatment appeared not to influence the results of PDT and were similar in both the cases and the controls. The proportion of patients with aneuploidy in addition to HGD in both groups prior to PDT was similar with a median number of 2cm biopsy levels with aneuploidy of 1 (range 1-4) in each group.

LGD found on histology following PDT was not shown to be indicative of future relapse in this study. LGD is a difficult diagnostic entity with poor agreement between pathologists (Lovat et al, 2006; Skacel et al, 2000) and it has also been shown that LGD poorly predicts progression to cancer at a rate of between 3-15% over five years (Reid et al, 2000; Sharma et al, 2006; Skacel et al, 2000). This also appears to be the case following PDT with little additional prognostic information being gained by considering LGD. This study does not suggest that changes to the follow up protocol for endoscopies can be soundly based around the presence or absence of LGD.

Aneuploidy, however, does appear to be able to predict relapse. Ninety percent of cases (9/10) were shown to have residual aneuploidy following PDT with all five of the patients developing cancer shown to have persistent or new aneuploidy following PDT. The patient that relapsed to HGD 38 months post therapy but was not shown to have residual aneuploidy has not developed cancer in a total follow up of 45 months. It may therefore be possible to defer follow up in the absence of aneuploidy post PDT for up to 5 years. Furthermore, only 10% of controls (2/20) were found to have aneuploidy post therapy. Both of these patients have relatively short follow up to date (24 and 26 months) and these two patients might relapse in the future. These data raise the possibility of image cytometry being used to define patient risk post ablative therapy and to potentially predict late relapse (OR 81, 95% CI 6.5-1017) defining a high risk group.

The number of samples processed for aneuploidy seems large but if proven to be predictive the pre-PDT biopsies would no longer need to be processed. Additionally only a single time point, between 2-6 months post therapy, might need to be assessed for aneuploidy every 4 or 5 years. The resulting reduction in the frequency of endoscopies for the majority of patients would provide significant financial savings by reducing the number of recommended endoscopies from 8 to potentially as few as 3. Patients free of residual aneuploidy can be reassured of a very low cancer risk in the following five years and also be freed of the burden of regular follow up. Late relapses to cancer remain a concern following all ablative therapies including PDT and occur in

up to 14% of patients. If these patients can be identified early they could undergo further treatment prior to relapse or be more intensively surveyed.

If fewer biopsies had been processed (only a posterior biopsy series for example) this would have resulted in only 7/10 cases being correctly identified and one patient who subsequently relapsed to cancer would not have been detected. This would be a worrying loss of sensitivity defeating the purpose of screening for residual aneuploidy post PDT. It would, therefore, seem necessary to perform image cytometry on both a posterior biopsy series and the pooled other biopsies collected from the same 2cm level of residual Barrett's post PDT.

Other studies have looked at small numbers of patients that relapsed late after PDT and examined multiple markers to define risk. Although persistent aneuploidy measured by image cytometry is accurate post PDT and its presence is associated with a high odds ratio (OR=81) it did not detect one patient who went on to relapse to HGD. It may be that a group of biomarkers may increase sensitivity further.

These results for aneuploidy need to be confirmed in a larger cohort of patients but could potentially significantly alter the need for regular surveillance endoscopies in the low risk group of patients post treatment. This may allow the targeting of follow up or even further treatment at the high risk group with residual aneuploidy.

## **5.6 Summary of the Validation of Image Cytometry in predicting future cancer risk in Barrett's Oesophagus**

The measurement of aneuploidy using image cytometry has not previously been accepted as a validated method in Barrett's Oesophagus. These studies have defined the diagnostic criteria for aneuploidy using ICM on a cut paraffin embedded section.

These criteria are based on those published for flow cytometry by the Seattle group but take account of the potential differences in the technology used. The potential errors corrected for by differing methodology included those resulting from processing paraffin embedded biopsies, cutting a 40micrometre section and collecting fewer nuclei per histogram. A small double blind study demonstrated that image cytometry appeared to measure aneuploidy as accurately as flow cytometry performed in Seattle.

This methodology was validated in a case control study which suggested that if residual aneuploidy persisted post PDT then the patient was at significantly higher risk of future relapse to HGD or cancer. This retrospective data analysis detecting aneuploidy prior to disease development fulfils the criteria for Phase 3 of biomarker development using ICM. Continuing biomarker development using image cytometry for the detection of aneuploidy predicting future cancer risk (a phase 4 prospective biomarker trial) poses significant problems in this unit. The first and perhaps most important consideration is the ethical question of not offering high risk patients treatment prior to cancer development. This might be justifiable for aneuploidy without HGD but causes some concern if HGD is found on histology. Additionally, all high risk patients with HGD on histology are offered minimally invasive therapy preventing patient analysis in a cohort for cancer development. In order for aneuploidy measured by ICM to be assessed in phase 4 of development a cohort of patients followed by another group would have to be sought and their archival baseline biopsies processed for aneuploidy.

Finally, detection of aneuploidy at UCH might allow the final step in biomarker validation; a phase 5 biomarker study. No phase 5 biomarker studies, with the exception of HGD, have to date been carried out. This study might involve randomising patients with aneuploidy but not HGD either to ablative therapy with PDT or to surveillance with the endpoint being the development of HGD or cancer.

## Chapter 6

# Introduction of Elastic Scattering Spectroscopy

## 6 Introduction of Elastic Scattering Spectroscopy

### 6.1 The Elastic Scattering of Light:

#### 6.1.1 *Mie Theory and Elastic Scattering Spectroscopy*

As described in Chapter 2.3.2, the elastic scattering of light is based on white light reflectance. Incident photons are back-scattered at the same wavelength from particles in matter. This was defined by Mie theory, and has been called the elastic scattering of light.

Gustav Mie in 1908 published his solution to the problem of elastic light scattering by homogeneous spherical particles. A particle, for the purposes of elastic scattering, can be defined as an aggregation of material that has a different refractive index to its surroundings. A common example of Mie Scattering in nature is the phenomenon that clouds appear white. Water droplets within the cloud have an approximate diameter of 20 micrometres so that all visible wavelengths are scattered equally resulting in the clouds appearing to be white. Furthermore, although Lord Rayleigh first described that the scattering of light is more intense at shorter wavelengths (blue) off small particles ( $<100\text{nm}$ ), Mie theory also explains why the sky appears blue and the sunsets red. When white light from the sun reaches the Earth the incident blue light is scattered more by the particles in the atmosphere than the red light of longer wavelength. Looking at the sky during the day, therefore, means that more light of shorter wavelength is scattered into your eye and the sky appears blue. As the sun sets and you look *directly* towards the sun the incident light has to pass through the atmosphere to your eye. Blue light is preferentially scattered away from your eyes and the sunset, therefore, appears red.

In biomedical optics, the scattering of photons has been shown to be an important event potentially with diagnostic value. ESS has been defined as the spectroscopic measurement of both the elastic scattering of light via Mie theory and the absorption of light by the tissue. To make matters more complex many different terms have been used to describe this spectroscopic collection of elastic light scattering with or without additional measurement of light absorption. The optical techniques, described to date, for measuring both elastic scattering and light absorption are Elastic Scattering Spectroscopy (Lovat and Bown, 2004) and Diffuse Reflectance Spectroscopy, (Georgakoudi et al, 2001) but for simplicity these phenomena will be referred to collectively as ESS from now on.



Light Scattering Spectroscopy (LSS) has also been described and is also a measure of elastic scattering and absorption but in this case only of singly backscattered photons. The measurement of this was achieved by only collecting light with the same polarity as that of the incident light. In multiple scattering events the polarity of light changes but after only a single scattering event the light retains its original polarity. This was termed 'polarisation', is a subset of ESS and its measurement will be referred to separately as LSS (Georgakoudi et al, 2001; Wallace et al, 2000). Finally, a more complex extension of LSS has been described and termed four dimensional elastic light-scattering fingerprint (4D-ELF) spectroscopy. This technique has been reported to measure the elastic scattering of photons, singly scattered light at very high angles (between 175-185° to the incident light). Additional polarisation-sensitive detection with this technique has been shown to provide an important advantage over unpolarised measurements because it allowed the operators to select returning photons from a specific depth of light penetration into tissue (Roy et al, 2004; Wali et al, 2005). This has been used to detect submucosal microvasculature as a marker of concurrent neoplasia in the colon (Roy et al, 2006; Siegel et al, 2006).

The disadvantages of the latter two systems, LSS and 4D ELF, are their complexity and expense. Additionally, at present 4D ELF spectroscopy cannot currently be used with 'through the endoscope' technology.

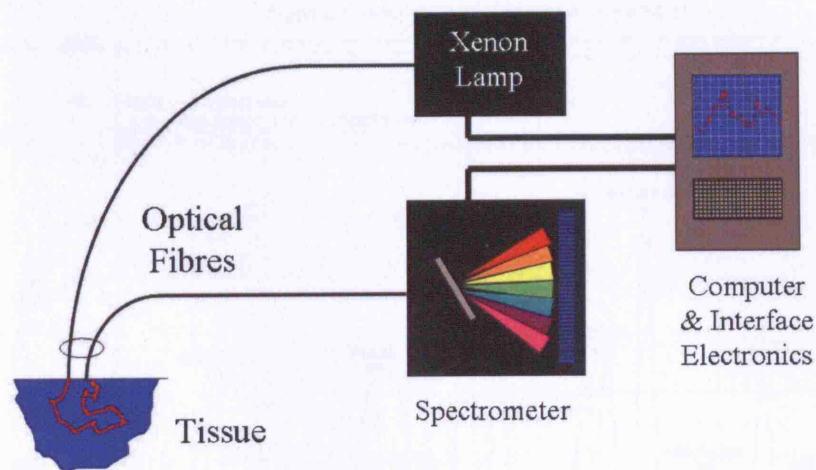
All of the work described in this thesis was performed using ESS with the principal focus being its translation into a clinically useful tool available to the endoscopist in the endoscopy room. The advantages of ESS over its more technological cousins are that it is cheap, robust with minimal and uncomplicated calibration, fast in measurement acquisition and is currently usable in the endoscopy room. These advantages mean that it could be moved to multicentre testing quickly if initial studies were successful. Its principal disadvantages are the potential lack of discrimination at micrometre resolution and, as with all of the technologies described, translational difficulties such as angle and pressure of spectroscopic acquisition *in vivo* and patient movement artifacts.

The rest of this chapter will be given over to descriptions of elastic scattering and light absorption in tissues, ESS hardware, ESS software and the current methods of statistical analysis.

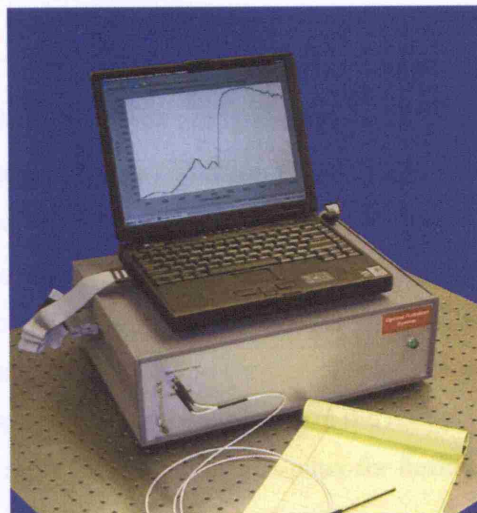
### 6.1.2 Hardware of the ESS System and Optical Fibres

The ESS system contains a pulsed xenon arc lamp, an optical probe and a spectrometer. These components are controlled by a computer which also records the spectra (Figures 6.1 and 6.2).

**Figure 6.1: Schematic of the ESS System**



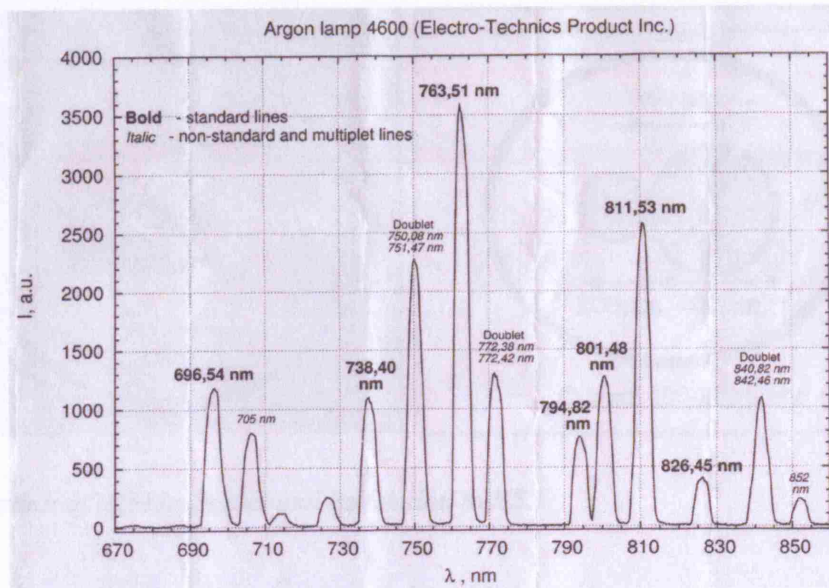
**Figure 6.2: Picture of the ESS System**



The xenon lamp (Perkin Elmer, Massachusetts, USA) was chosen because it effectively has no warm up time. The lamp produces visible light between 320-920nm and it has a relatively constant output across this spectrum. The spectrometer (S2000, Ocean Optics, Florida, USA) was calibrated against an argon lamp (Electro-Technics Product Inc, Illinois, USA) which produced a

series of sharp well defined peaks of known wavelengths. These sharp peaks used for calibration are shown in figure 6.3. This allowed accurate calibration for the spectrometers light wells. The lamp, spectrometer and their power supply were mounted in a briefcase size unit which was portable and could be easily connected to a laptop computer. For safety reasons ultraviolet B & C (100-315nm) light were filtered out to avoid the risk of cellular DNA damage.

**Figure 6.3: Argon lamp peaks and the wavelengths used for spectrometer calibration**

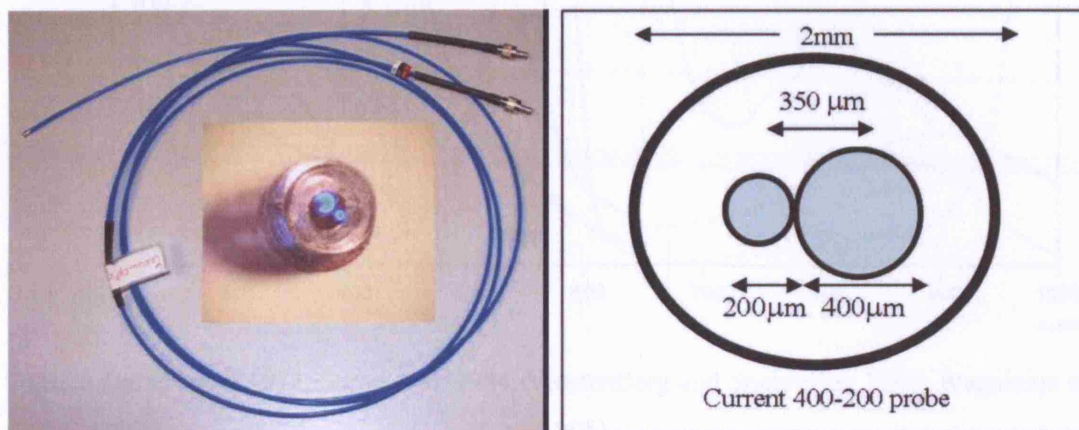


In operation, short pulses of white light from the xenon arc lamp were directed through a flexible optical fibre (400µm diameter) which was gently placed in contact with the tissue under examination. In the same optical probe a collection fibre (200µm diameter) collected the light back-scattered from the tissue. These two fibres were encased in an outer sheath and the fibres were separated by a distance of 50µm with a fixed centre-centre separation distance of 350 µm (CeramOptec Industries, Bonn, Germany). It has been shown that the smaller the optical fibre separation the greater the sensitivity of ESS to the size of scatterers in the tissue (Mourant et al, 1997). The light was propagated into the spectrometer in the returning optical fibre and the outputted spectrum of light recorded in a laptop computer for further statistical analysis. In total each reading took less than 200 milliseconds to perform.

In summary, ESS is a fast, real-time, in vivo technique which should be able to detect changes in the physical properties of cells. The data below examines whether ESS in principle could detect the changes in the physical properties of tissue which are closely related to both HGD and Aneuploidy.

**ESS Optical Fibres:** The fibre assembly was housed in a plastic sheath (outer diameter 2.0 mm) which was passed into the oesophagus via the biopsy channel of a standard endoscope. Figure 6.4 The fibre was sterilised with the endoscope.

**Figure 6.4: Picture of optical fibre (400-200 $\mu$ m) with calibration pot and schematic cross-section**



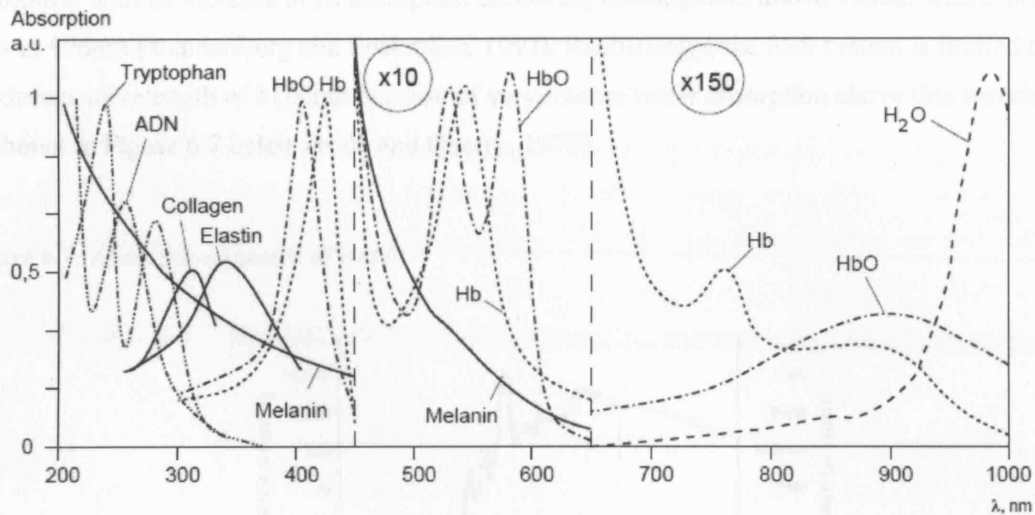
### 6.1.3 Absorption of light in tissues and its relation to ESS

Light from 350nm-450nm (in the ultraviolet region) has been shown to be mainly absorbed by haemoglobin. This absorption feature, which dominates the spectrum of light at these wavelengths, has been called the Soret band with peak absorption at 414nm. Above 400nm there is enough light produced by the xenon lamp and enough transmission of this light through tissue to allow for optical measurements to be collected.

In the visible spectrum (450nm-700nm) oxy- and deoxyhaemoglobin have been described as the biggest absorbers of light. This absorption occurs between 500-630nm with the peak for oxyhaemoglobin and deoxyhaemoglobin at 540nm, and a second peak for oxyhaemoglobin at 575nm. These absorption features have been termed the Q Bands of haemoglobin. The relative absorption strengths of these chromophores and other principle tissue chromophores have been compiled together in figure 6.5 below. It remains uncertain, however, whether the effect of this absorption is going to be important for diagnostic purposes (Roy et al, 2006; Siegel et al, 2006; Wali et al, 2005) and, therefore, it remained part of our 'diagnostic window' throughout the experiments in this thesis.



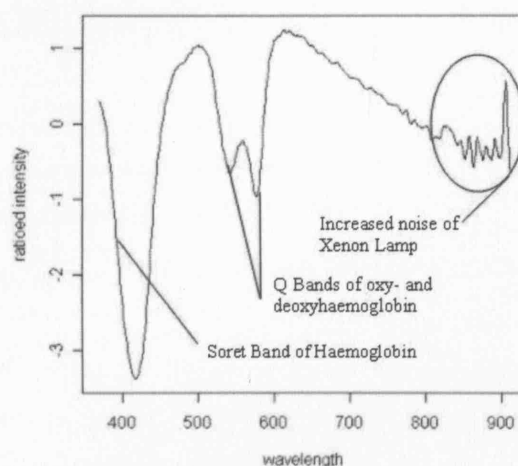
**Figure 6.5: Compilation of absorption spectra of principal chromophores in tissue**



(Hale and Querry, 1973; Sarna et al, 1984; VandenBerg and Spekrijse, 1997; Wagnieres et al, 1998)

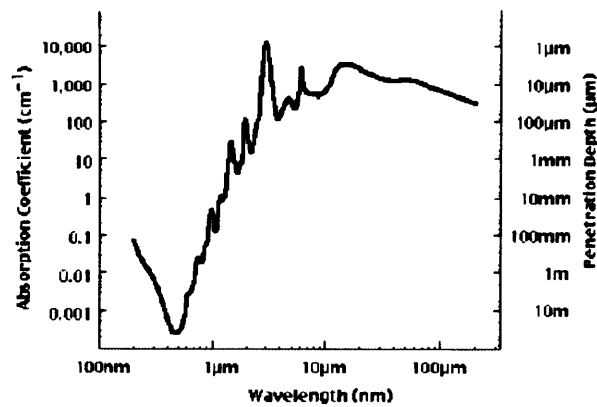
Haemoglobin, as the principal chromophore, was known to have a marked effect on the ESS spectra. The Soret band of haemoglobin and the Q bands of oxy- and deoxyhaemoglobin are demonstrated on the example ESS spectra in figure 6.6 below which was collected in vivo using our ESS system from a patients Barrett's Oesophagus. The strong absorption of haemoglobin in the visible wavelengths of blue and green light is the reason that blood appears as a vivid red colour.

**Figure 6.6: An example ESS Spectra (smoothed, reduced and normalised) with the Soret and Q bands marked**



In the infrared (wavelength  $>900\text{nm}$ ), the absorption by water is the strongest contributor to tissue absorption with an increase in its absorption exceeding haemoglobin above  $930\text{nm}$  with a small peak at  $976\text{nm}$  (VandenBerg and Spekreijse, 1997). Realistically, the ESS system is limited to a maximum wavelength of  $1100\text{nm}$  because of very intense water absorption above this wavelength as shown in Figure 6.7 below (Hale and Querry, 1973).

**Figure 6.7: Absorption spectra of water**



(Hale and Querry, 1973)

#### ***6.1.4 ESS and Particulate scattering in biological tissues***

One of the principal cellular scatterers for ESS is the nucleus. Several properties of the nucleus have been shown to change the pattern of elastic scattering. The first and potentially simplest to measure is nuclear size which has been strongly positively correlated with high angle scattering (Drezek et al, 1999; Drezek et al, 2003a; Gurjar et al, 2001; Mourant et al, 2000; Wallace et al, 2000; Zonios et al, 1999). Secondly, nuclear density has also been shown to change the ESS spectra (Zonios et al, 1999) and nuclear chromatin content (a phenomenon closely related to aneuploidy) has been shown to affect the spectra of singly scattered light (Backman et al, 2000; Gurjar et al, 2001) and high angle scatter in ESS (Mourant et al, 2000). Finally, the nucleus:cytoplasmic ratio, along with other intracellular changes, has been shown to have a significant effect on elastic scattering in Monte Carlo modelling (Drezek et al, 1999).

Another group of principal scatterers in cells are the mitochondria. These intracellular organelles are about 1  $\mu\text{m}$  in length and they are composed of many folded lipid membranes called cristae. The density of lipid:water interface within the mitochondria make them especially strong scatterers of light. ESS has been shown to be able to detect changes in the number of mitochondria in Monte Carlo modelling of light scatterers in tissues (Drezek et al, 1999), in cellular suspensions (Mourant et al, 2000) and clinically in the cervix (Drezek et al, 2003b). The ESS spectra is altered by changes in the refractive index of the cell which in turn has been related to changes in all of the intracellular components (Mourant et al, 2000).

Finally, the cellular packing density has also been shown to change the elastic scattering of light in light scattering spectroscopy (Wallace et al, 2000).

#### ***6.1.5 ESS and precancerous changes in tissue***

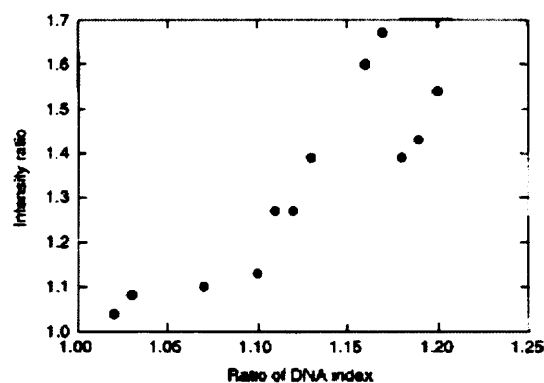
ESS is sensitive to the changes in the physical properties of tissue described above. Do these physical properties, however, change with dysplasia and aneuploidy?

Firstly with regard to the nucleus, our image cytometry data has shown that the size of the nucleus changes by up to 50% in aneuploidy compared to diploid nuclei (section 5.4.4). This has also been shown to be the case for LGD and HGD (Polkowski et al, 1998; Zonios et al, 1999).

Additionally cellular packing and nuclear size are two of the previously modelled parameters of ESS (diffuse reflectance) in the detection of HGD (Wallace et al, 2000). Nuclear:cytoplasmic ratio is a criteria used by histopathologists for the diagnosis of HGD.

As already discussed, changes in high angle scatter with ESS have been shown in cell suspensions not only to be associated with nuclear size but also with changes in nuclear chromatin content (Mourant et al, 2000). Figure 6.8 demonstrates that as the ratio of the average DNA content (DNA index) between two cell suspensions increases so does the intensity of high angle scattering (between 110° to 140°). Both nuclear size and chromatin content are features of aneuploidy and to an extent HGD.

**Figure 6.8: Ratio of scattering intensities (110° to 140°) between two cell suspensions with different DNA indices**



(Mourant et al, 2000)

There are other ultrastructural changes associated with HGD and aneuploidy in Barrett's Oesophagus. On electron microscopy distension of the rough endoplasmic reticulum is associated with both aneuploidy and HGD (Levine et al, 1989a) as are increased accumulation of cytoplasmic glycogen aggregates such as the Golgi apparatus and secretory granules (Levine et al, 1989b). Additionally, the mitochondria in tumour cell lines have been shown to change with respect to their numbers, size and shape when compared to normal controls (Modica-Napolitano et al, 2007). In the upper aero-digestive tract increases in the number of mitochondria have been associated with increases in the grade of dysplasia (Kim et al, 2004).

In theory ESS should, therefore, be sensitive to changes both directly related to HGD and aneuploidy (nuclear size, density, changes in the cellular packing density and changes in the



nucleus:cytoplasmic ratio) and also indirectly via changes in the number of mitochondria, the rough endoplasmic reticulum and Golgi apparatus.

## **6.2 Calibration**

The system has inherent variation between patients which can account for differences in the spectra acquired. These variations occur in the light source, spectrometer, optical fibre transmission, interconnects within the ESS system and fibre coupling. For example these changes occur due to minute differences in the exact positioning of the optical fibre and interconnects and the temperature of the endoscopy room.

In order to account for these changes a white reference spectrum is recorded from Spectralon™ (Labsphere, New Hampshire, USA). The elastic scattering properties of Spectralon demonstrate a flat spectra between 250-1000nm and therefore the spectra measured is due to the ESS system itself. This reference reading can be removed prior to statistical processing allowing for accurate comparison between patients, optical fibres and measurements recorded on different days.

## **6.3 Dynamic Dark Subtract**

The ESS system automatically recorded the ambient light within the oesophagus from the endoscope light source immediately before any reading was made. The measurement specific exogenous light was therefore controlled for and removed prior to analysis.

#### **6.4 Autoranging**

This allowed for the maximum amount of the 'dynamic range' of the spectrometer to be used. Dynamic range can be defined as the maximum counts of light that any individual well in the spectrometer could measure. The spectrometer in the ESS box used in these studies had 1801 wells between 320-930nm and each well measured up to 4095 counts. The ideal number of counts at the most intense part of the recorded spectrum was therefore about 95% of this range (ie 3900 counts). Above 95% of the maximum capacity of light measurement the spectrometer became less reliable with the amount of light counted in a less linear way.

Autoranging is a computer process which allowed a measurement to be repeated if the maximum returning counts of light were less or more than a pre-determined level (for example low limit 80% of the dynamic range or 3275 counts and upper limit 95% of the dynamic range or 3900 counts). This repeat measurement was performed with an increased or decreased amount of light being shone into the target tissue with a consequent change in the intensity of back-scattered light.

This process was repeated until the measurement fell into the desired range. The speed of spectral acquisition was so fast that the operator was almost unaware of this process occurring with the total acquisition time less than 200 milliseconds.

## 6.5 Analysis of Results

Throughout all the studies in this thesis 4 ESS spectra were collected from each site. This was originally performed to ensure spectra taken very rapidly after each other from the same site were consistent. This has been examined by Miss Ying Zhu at the laser centre and a brief synopsis of her results is presented in section 7.5. From the perspective of result analysis all spectra from one site were always included in either the training or test set.

### 6.5.1 Statistical Model Generation

The ESS spectra were cleaned before analysis. Initially each spectrum was made up of 1801 intensity points spanning the wavelength range 320nm to 920nm. Spectra were smoothed using a standard smoothing technique called the Savitzky-Golay method in order to reduce the random adjacent wavelength variation or 'noise'. With advice from Professor Tom Fearn, Head of Statistics, UCL, we elected to use heavier smoothing above 620 nm, where the signal-to-noise ratio was greater. Below 400 nm and above 800 nm, the signal-to-noise ratio of the spectra was too low for analysis. The Xenon arc lamp has a lower light output at these wavelengths and the absorption of biological tissue was greater. Only the window between 400-800 nm was therefore used in the analysis. The spectra were then normalised allowing for more accurate shape comparison. This was performed by setting the mean intensity of each spectrum to zero and a standard deviation of 1 by subtracting its mean intensity and dividing each spectrum by its standard deviation. A principal component analysis (PCA) and then linear discriminant analysis (LDA) were performed.

#### *Principal Component Analysis and Linear Discriminant Analysis*

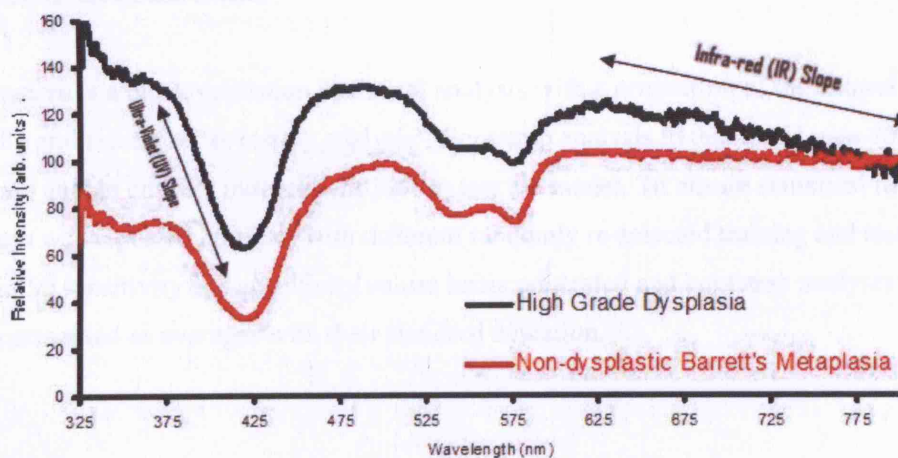
A linear discriminant analysis can be used to maximise the discrimination between the two groups (described in the paragraph below) but using all the remaining points in the spectrum would cause a problem with collinearity in a LDA. Collinearity results from the high correlation between data points in the spectrum. One assumption made for LDA was the independence of all the variables used and, therefore, a PCA was performed first. A principal component analysis results in the ordered feature selection of the dataset after an orthogonal linear transformation has been used. Principal component 1 would, therefore, represent the greatest variance of the data, principal component 2 the second greatest variance and so on. The data points were reduced into these principal components (PCs) which captured the variability of the spectrum with virtually no loss of information.

It was possible to overfit the training algorithm if excessive numbers of PCs were used in the LDA. The algorithm generated would then only work for that specific dataset and the results would not be generalisable to other data or the general population. This has been termed 'overfitting'. Overfitting becomes less likely the fewer the PCs used especially if the number of PCs used was much smaller than the number of spectra. For example, in the most straightforward of examples; any two spectra can be divided into 2 groups with complete accuracy in an LDA if 1 PC were used and any three spectra could be divided into 2 groups if 2 PCs were used.

It was, therefore, statistically possible to accurately distinguish  $N$  different readings into two groups if a linear discriminant analysis containing  $N-1$  PCs was used. The question that remained was "What is the ideal number of PCs to choose and at what number of PCs does the risk of overfitting increase?" This is discussed in section 6.6.4.

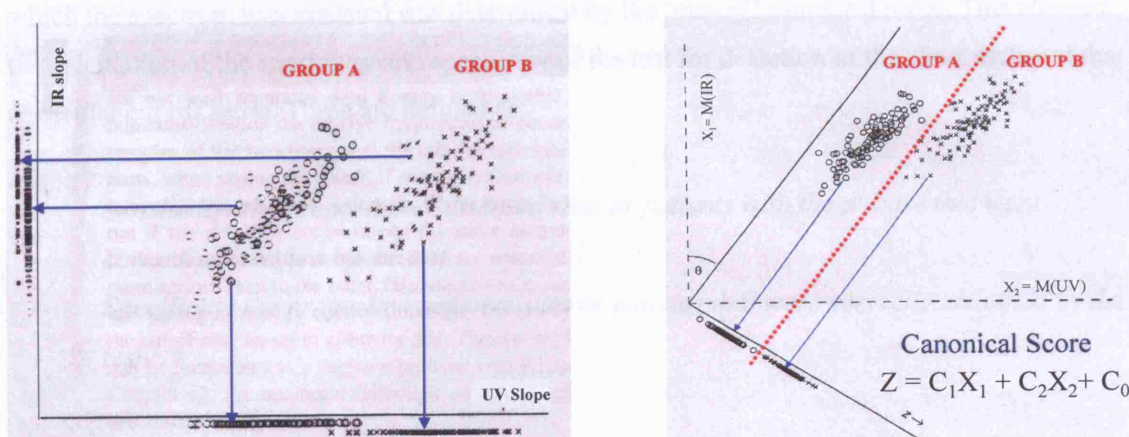
Once the number of PCs to be used was decided, a linear discriminant analysis (LDA) was then applied to these principal components to maximise the discrimination between the two groups. LDA has been described as a linear rotation of the original axes with the PCs of the spectra projected onto this axis which resulted in the generation of the first canonical score. In the discrimination between two groups only one canonical score was used (multiple scores being used in discrimination between multiple groups). The canonical score obtained was a continuous variable. In the example below in figure 6.9, two imaginary PCs were used; the Ultra-violet (UV) slope and the Infra-red (IR) slope.

**Figure 6.9: ESS Spectra with two imaginary PCs marked, the IR and UV Slopes**



These two PCs were plotted on to the x and y axes respectively and two imaginary datasets (group A without disease and group B with disease) displayed. In the figure 6.10 the graph on the left displayed the discrimination if the horizontal or vertical discrimination were to be used which only offered some differentiation but if the axes were rotated by  $\theta$  degrees, as seen in the graph on the right, in a LDA then the discrimination would be significantly improved.

**Figure 6.10: Illustrative figures of an imaginary two dimension dataset with simple analysis compared to LDA rotating axes to improve discrimination**



#### *Types of statistical analysis for model generation*

Leave one out cross validation statistical analysis or 'jackknife analysis' trains the algorithm on all the data except one point which is then tested. All spectra collected from a single site are confined to either training or testing to maintain independence of training and test sets. This is repeated until all points have been tested.

The alternative is a block validation statistical analysis with a proportion of the dataset assigned for training and testing or 'bootstrap analysis'. Bootstrap analysis in this thesis uses 70% of the data to train and an entirely independent 30% to test the model. To ensure statistical robustness, this process was repeated 50 times with different randomly re-selected training and test sets. This resulted in 50 sensitivity and specificity values being generated and bootstrap analyses are therefore presented as averages with their standard deviation.

### 6.5.2 Model Validation

Bootstrap and Jackknife analyses allow for each statistical algorithm (or model of the dataset) generated to be tested to give an estimation of the accuracy of discrimination. This has been termed ‘model validation’. Multiple spectra were taken per site and all of these spectra from any given site were kept together, in either the training set or the testing set, to prevent any bias being introduced through non-independence of data. Using the algorithm created with linear discriminant analysis, a canonical score (the output from the LDA) for each test spectrum was determined. This allows the spectrum to be assigned into one group or the other. The group into which the spectrum was assigned was determined by the ‘cut-off’ canonical score. This allowed the calculation of the sensitivity and specificity of the test for detection of the abnormality at that particular cut off score. These are defined as:

***Sensitivity:** the proportion of diseased sites or patients with the disease that were correctly identified by the test*

***Specificity:** the proportion negative sites or patients that were correctly identified by the test.*

The cut off score could be adjusted to favour either sensitivity or specificity or to optimise the overall accuracy of the test. A Receiver Operating Characteristics (ROC) curve was obtained by plotting the sensitivity and 1-specificity of the algorithm for the whole range of ‘cut-off’ canonical scores. The area under the ROC curve (AUC) represented the overall accuracy that an algorithm can be expected to produce.

A ROC curve was obtained for both *per spectra* and *per site* analyses. In the former all spectra were considered for the generation of the ROC curve but in the per site analysis only the spectra with the highest score at that site counted. In Barrett’s Oesophagus it was felt more important not to incorrectly classify high risk patients as low risk because this might have left these patients exposed to increased cancer risk. If low risk patients were falsely identify as high risk this would have only resulted in a biopsy being collected from that site and being processed in the normal way. In order to reduce the risk of missing high risk sites all the algorithms and subsequent methodology were aimed at loading towards sensitivity. In per site testing, therefore, only the highest score in the group of spectra from that site was counted in contributing to the risk for that site. Cut off criteria were also decided by loading towards high sensitivity at the expense of specificity.

A simple cut-off was decided and all patients with a higher or 'positive' score were then placed into the same group. This, however, potentially wasted some of the information provided by a continuous output variable. In simple prospective testing of the algorithm therefore in the following studies it might be possible to assign a varying positive predictive value (PPV) to different canonical scores. A negative predictive value (NPV) might also be estimated for the whole examination.

***Positive predictive value:*** the proportion of patients with a positive result to the test who were correctly diagnosed.

***Negative predictive value:*** the proportion of patients with a negative test result who were correctly diagnosed.

These measures are dependent on the accuracy of the screening test and the prevalence of the disease in a surveillance population. NPV and PPV are felt to be of more relevance to a clinician because they specifically define a particular patient's risk not the abstract overall accuracy of an investigation. The PPV and NPV of ESS were examined further in the results section of the following chapter (7.4).

### **6.5.3 Simple Prospective Testing of ESS Model**

The ultimate goal of testing was to assess the predictive ability of a predetermined algorithm on an entirely separate test set running the analysis just once to determine the sensitivity and specificity if this algorithm had been used in the clinical setting. Statistically this was simpler because the algorithm was calculated in advance and new spectra just analysed one by one with a canonical statistical score generated for each site. A simple prospective test also negated difficult to define statistical risks of algorithm generation, such as overfitting (see below in 6.6.4), and removed the potential for unknown biases being introduced at the model generation stage.

Simple prospective testing allowed an entirely independent sensitivity, specificity, PPV and NPV to be calculated free from many biases. The criticisms that can be laid at this kind of testing were really methodological and included factors such as generalisability (eg whether the data were collected from patients who were representative of the whole population).



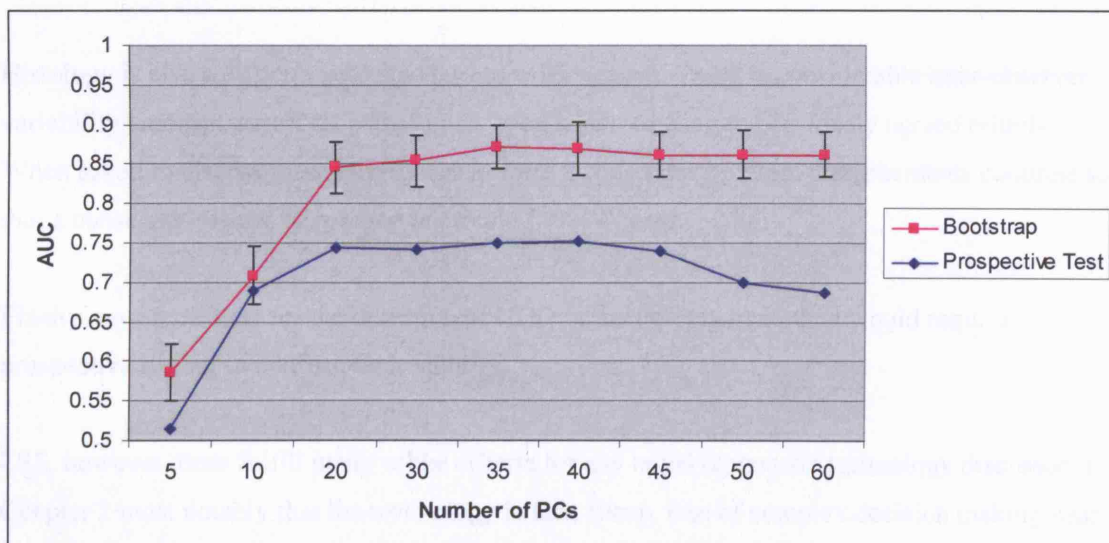
#### 6.5.4 The number of principal components chosen for LDA

The number of principal components chosen for the linear discriminant analysis could have affected not only the results of algorithm generation but also of prospective testing. Careful consideration was required to ensure the number chosen was appropriate. As mentioned above in section 6.5.1 it was possible to overfit the training algorithm if the number of PCs used in an LDA approximated to the number of different spectra or sites. The question “What was an optimal number of PCs to use in a LDA without overfitting the data?” needed to be addressed.

In a jackknife or bootstrap analysis it was possible to deliberately overfit the training algorithm by continuing to increase the number of PCs used. Increasing the number of PCs beyond the optimal point could have improved the initial results but when this algorithm was applied prospectively to an independent dataset the accuracy fell.

Figure 6.11 below demonstrated the principle of overfitting. In the training bootstrap algorithm developed from over 200 sites for detection of high risk sites in BO the AUC improved rapidly up until 20 PCs and then only very marginally thereafter flattening out at more than 45 PCs. In a test for potential overfitting, an independent prospective test set was then used and the AUC plotted. This AUC again improved until approximately 20 PCs, flattened off and then started falling probably due to overfitting after 40 PCs. It therefore seems reasonable to pick the number of PCs in-between 20-40 and 30 PCs was therefore chosen for all testing from this point forward.

**Figure 6.11: Number of Principal Components affects the Area under the ROC curve (AUC)**





Ying Zhu, the PhD student in statistics at the laser centre, has shown that only 3.4% of the spectral variation is due to differences in the mucosa that distinguish HGD from non-dysplastic Barrett's. The other 96.6% of the variation in vivo can be explained, and replicated ex vivo, by biological variability between subjects, and changing the angle and pressure of the optical probe during measurement acquisition. This raised an important but far more statistically complex second question 'Can we remove this non discriminatory variability (noise) from our in vivo spectra and either improve accuracy and/or use fewer principal components?' This will be discussed in Chapter 7.5.

## **6.6 Discussion**

Elastic Scattering Spectroscopy like all current novel optical technologies has both advantages and disadvantages. It provides results in under a second, is cheap, simple to use and removes the need for operator interpretation. The drawbacks are that it is unlikely to be accurate enough to replace standard histology reducing it to the targeting of biopsies for further histological processing in a surveillance population. Additionally, as a point measurement ESS needs to be fast enough to survey an area of the gut reliably and efficiently. Its use through an endoscope in a living patient also presents many methodological issues including potential lack of true matching of optical measurements and physical biopsies from exactly the same point and changes in the angle and pressure of measurement acquisition. These technical problems are discussed in detail in section 7.4.

Histology is also a difficult gold standard to work against. There is considerable inter-observer variability amongst expert GI pathologists even when working to previously agreed criteria. When asked to discuss these differences to form a consensus opinion, disagreements continue so that a consensus cannot be reached in around 17% of cases.

Finally any algorithms for the detection of HGD or aneuploidy generated would require prospective testing to confirm their validity.

ESS, however, does fulfill many of the criteria for any novel diagnostic technology discussed in Chapter 2 most notably that the technology is fast, cheap, free of complex decision making near the patient, does not require extended periods of specialist training and is compatible with

existing endoscope technology. There are also good scientific data to support the concept of ESS for the detection of HGD and aneuploidy in BO. In chapter 7 the results of clinical testing are presented.

## Chapter 7

# Elastic Scattering Spectroscopy for Surveillance in Barrett's Oesophagus

## 7 Elastic Scattering Spectroscopy for Surveillance in Barrett's Oesophagus

### 7.1 Introduction

ESS has been used to study human tissues other than Barrett's Oesophagus for the diagnosis of cancer in the breast, sentinel lymph nodes, bladder and mouth (Mourant et al. 1995;Chicken et al. 2004;Johnson et al. 2004;Sharwani et al. 2006). The most studied of these to date is breast cancer where ESS spectra have been collected from 72 histological sites within the breast and in 54 sentinel lymph nodes with sensitivities ranging from 69% to 85% and specificities from 58% to 93% (Chicken et al. 2004;Johnson et al. 2004). The analysis of sentinel lymph nodes for metastases on a per node basis showed a sensitivity of 75% and specificity of 95% (Johnson et al. 2004) comparable to touch imprint cytology with a sensitivity of 75% and specificity of 98% (Lee et al. 2003). Intra-operative fresh frozen histological analysis of sentinel lymph nodes, the current gold standard has been shown to have a sensitivity of 65-87% and specificity of 99-100% (Brogi et al. 2005). More recent data for ESS in the diagnosis of sentinel node metastases has shown excellent results by using an automated scanner to examine a larger area of the node. This scanner collected 400 spectra (in a 20x20 grid) every 0.5mm over a 1cm<sup>2</sup> area across the lymph node. In this prospective test of the sentinel node metastasis algorithm a per node sensitivity of 74% and specificity of 96.5% was achieved and the results are comparable, if a little less specific than the accuracy of touch imprint cytology and fresh frozen histology quoted above.

As described in chapter 6, ESS should be sensitive to the ultrastructural changes in tissue associated with both HGD and aneuploidy. The ability to detect aneuploidy in BO is of importance because the Seattle group have suggested that combining the biomarkers HGD and aneuploidy could potentially offer greater predictive ability for those patients going on to develop cancer. Additionally, up to 87% may be freed from surveillance for at least 5 years (Rabinovitch et al. 2001;Reid et al. 2001;Reid et al. 2000b). Unfortunately, the measurement of aneuploidy by either image or flow cytometry is too labour intensive and expensive and is, therefore, currently not deliverable into routine clinical practice. Furthermore, unless standardised and painstaking methodology is applied with flow cytometry (such as the addition of a 5 minute manual trituration step per sample to prevent nuclear aggregation immediately prior to the samples processing through the flow cytometer) then errors have occurred (Reid et al. 1992;Alanen, Joensuu, and Klemi 1989). These reasons have prevented its use outside research centres and the incorporation of flow cytometry for aneuploidy into general hospital use. If a fast, reliable, *in vivo*

method such as ESS could be shown to detect aneuploidy in addition to HGD during routine surveillance endoscopy, a more accurate definition of those at high risk could be made, potentially improving patient management.

To make matters worse HGD has been shown to be ‘patchy’ throughout BO (Reid et al. 1988) with only an average of 1.3 cm<sup>2</sup> out of 32 cm<sup>2</sup> containing the abnormality (Cameron and Carpenter 1997). Additionally, only 1.3% of patients per year develop HGD (Sharma et al. 2006) with an average of just 15% (2.5/17) of those biopsies taken displaying HGD (Lovat et al. 2006). The estimated number of biopsies required, therefore, to diagnose one patient with HGD is >600 biopsies from over 75 procedures. In the face of these estimates it is hardly surprising that serious questions have been raised as to the value of Barrett’s surveillance (Garside et al. 2006). It was hypothesised that ESS could not only more accurately diagnose patients at high risk but could also reduce or in some cases remove the need to process so many biopsies for HGD and aneuploidy improving the current appallingly low diagnostic yield.

#### ***7.1.1 So what is required for a novel endoscopic technology to improve the surveillance of Barrett’s Oesophagus?***

A fast, robust, cheap technique which could be used through standard endoscopes without the need for extra endoscopist training and not subject to inter-operator variability would be suitable. Additionally, it should be able to more accurately define a patient’s future cancer risk than standard histology alone, removing the need to take or at least reduce the large number of biopsies currently taken for histology. This technique should also ideally allow a greater area of the patients Barrett’s segment to be examined. ESS might fulfil many of these criteria.

## 7.2 Overall Aim of this Chapter

The good theoretical evidence that ESS would be able to detect both HGD and aneuploidy in Barrett's Oesophagus was encouraging but the improvements in Barrett's surveillance described above were a challenging set of criteria. The ability of any optical technology to detect aneuploidy, a biomarker that is not currently possible to diagnose in clinical practice, would be a huge advantage but this advance must not come at the expense of other clinically important factors such as the diagnosis of HGD and the length of time required to complete the endoscopic procedure.

The aims of this chapter were to provide evidence that ESS was capable of:

- Detecting and potentially removing unwanted squamous spectra prior to analysis.
- Detecting both HGD and aneuploidy in Barrett's Oesophagus not only in algorithm development but also in prospective testing.
- Examining if the canonical score, the statistical output from the algorithm, could be utilised in a more effective way than just a simple binary outcome of risk.
- Performing a proof of concept study, prospectively testing our statistical algorithm, to examine if ESS was accurate and fast enough without inter-operator variability to adequately survey patients Barrett's Oesophagus in a surveillance population.

## 7.3 Clinical Studies of ESS

### *7.3.1 Matched biopsies for the detection of Barrett's mucosa compared to readings collected from squamous mucosa*

#### **Introduction and Aims**

Barrett's oesophagus is normally easily visualised at endoscopy and biopsies subsequently targeted to this visible lesion. It was important however for ESS to be able to detect Barrett's as opposed to squamous mucosa for two potential reasons. The first was to prove that ESS could detect this macroscopically visible abnormality prior to ESS being tested on more subtle microscopic mucosal abnormalities such as HGD and aneuploidy. The second concern was that in any statistical process with a binary outcome of high risk (eg HGD or aneuploidy) or low risk (no dysplasia/LGD and no aneuploidy) the inclusion accidentally of any third diagnostic entity (in this case squamous mucosa) could have resulted in high false positive rates entirely by chance.

The aims of this section were therefore to assess:

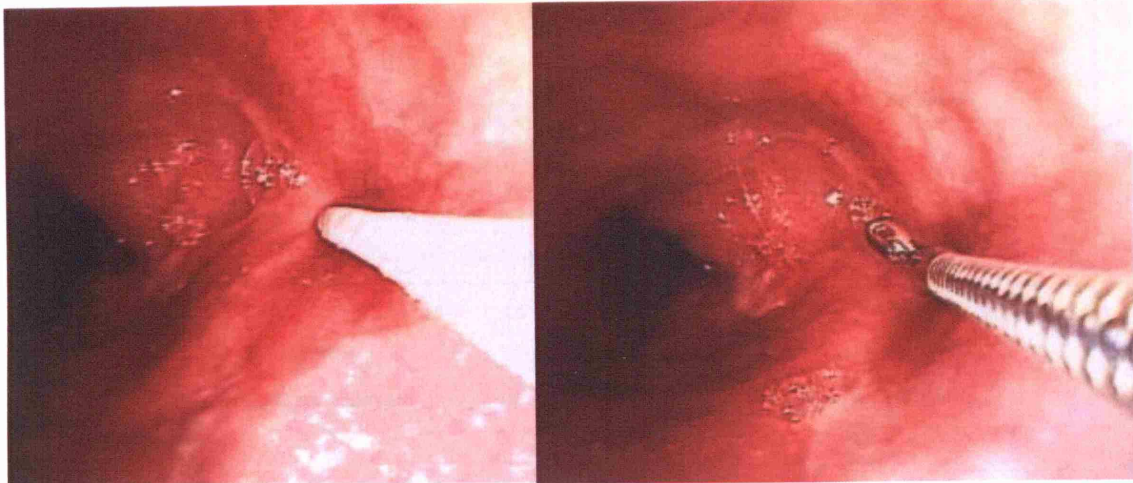
- If ESS could differentiate squamous from Barrett's mucosa (including inflammation and dysplasia if present) *in vivo* in both algorithm generation and independent prospective testing.
- Whether the accuracy of discrimination was high enough for automatic spectra exclusion if squamous mucosa proved to have a high false positive rate for HGD or aneuploidy.
- To form a test set comprised of squamous mucosa to analysis in any algorithm later developed for the diagnosis of HGD.

#### **Methods**

**Methodology for Matched Optical and Histological Biopsies:** During routine endoscopy for Barrett's surveillance, ESS optical measurements were taken and immediately followed by biopsy for histology from the same site. The tip of the optical probe was placed in gentle contact with the mucosa and the lamp and spectrometer were triggered via the keyboard of the laptop computer or an attached foot-pedal. After gentle pressure from the optical fibre a small red mucosal artifact is left for a short time and care was taken to ensure the subsequent biopsy was taken from this site. Figure 7.1.

The biopsy was then processed for histology in the standard way for histology.

**Figure 7.1: Matched Optical Measurement with Biopsy for Histology**



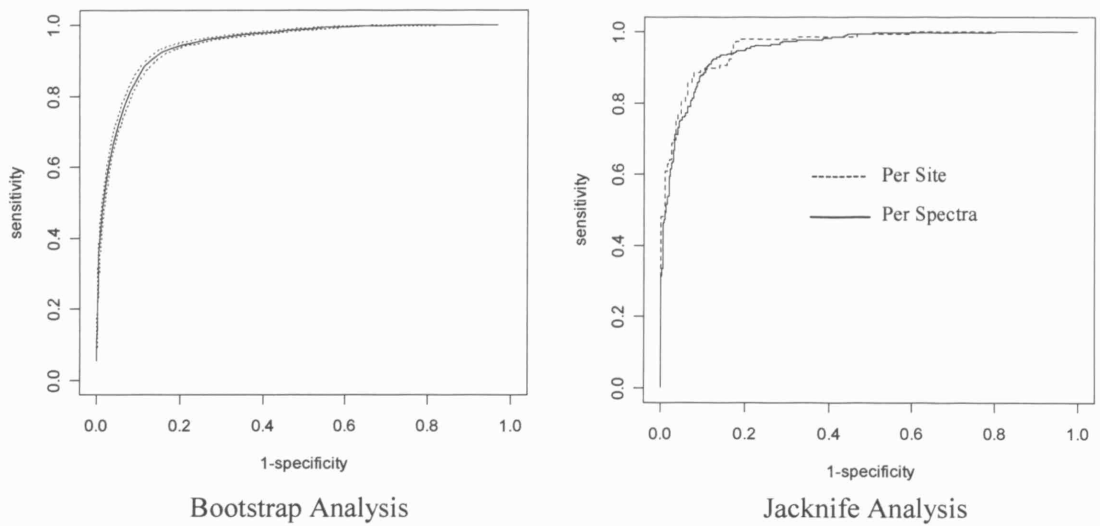
**Methods for algorithm development:** Algorithm generation for discrimination between squamous and Barrett's mucosa *in vivo* through the endoscope was undertaken. Matched optical and histological specimens were collected from Barrett's mucosa and all Vienna grades in Barrett's Oesophagus were included. These Barrett's optical measurements were compared to squamous readings taken 2cm more proximal to the Barrett's segment with single biopsies taken from each level of squamous mucosa for histological confirmation. All squamous sites were included in the analysis irrespective of degree of inflammation or degree of dysplasia if present. The performance of this algorithm was then tested in a prospective test of matched sites of squamous and columnar mucosa using the same methodology.

## Results

Two thousand one hundred and forty three optical measurements were collected for algorithm generation from 543 sites in 60 patients. Three hundred and five sites were from Barrett's mucosa and 238 sites from squamous mucosa. Both a block validation (Bootstrap) and a leave one out cross validation (Jackknife) statistical analyses were performed as described in Chapter 5 with virtually no difference in the accuracy reported between these two techniques. The Area Under the ROC Curve (AUC) in the Bootstrap analysis was 0.95 and 0.96 in a Jackknife analysis. These results displayed excellent discrimination. See figure 7.2.



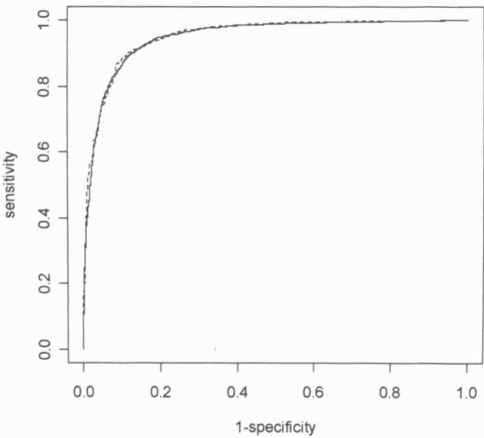
**Figure 7.2: ROC Curves of Bootstrap and Jackknife analyses of distinguishing Squamous from Barrett’s mucosa**



An independent prospective test of this squamous versus Barrett’s mucosa algorithm was performed on ESS measurements collected *in vivo*. The training set above was used on a prospective independent test set of 40 patients with 2245 spectra collected from 393 Barrett’s sites and 168 squamous sites.

The AUC continued to show excellent discrimination between squamous and Barrett’s mucosa at 0.95. If an equal balance was placed on both sensitivity and specificity for detecting Barrett’s mucosa then these data show a sensitivity and specificity of 90%.

**Figure 7.3: Simple prospective test for detecting Squamous from Barrett’s mucosa**



## Discussion

These data show that ESS had a high degree of accuracy in vivo for the detection of Barrett's Oesophagus when compared to squamous mucosa. Although this was an important step to prove the potential of ESS, the endoscopist would usually be able to adequately visualise a patient's Barrett's segment and, therefore, these results are likely to be of limited practical value. These data have allowed the formation of a squamous test set for testing for a false positive rate with a high risk algorithm developed in the future. If squamous readings were proven to be important false positives in any subsequent analyses then the accuracy of these results would allow automated removal of squamous spectra prior to subsequent analysis of Barrett's sites for future risk. All squamous and columnar sites were included irrespective of inflammation or dysplasia. This could be considered a weakness of the study but answers the clinical question regarding differentiation between all forms of squamous and columnar mucosa.

The sensitivity and specificity for the detection of Barrett's mucosa can of course be changed with a preference to either measure. It might seem sensible to consider that we do not want to exclude any columnar sites and therefore load towards sensitivity but were it to become possible to collect real-time measurements then another site within the 'apparent' Barrett's segment could be collected if the ESS software rejected the first site. Provided this did not lead to the exclusion of any high risk Barrett's sites then loading for specificity could be considered the correct option.

This careful decision must relate to the impact of accidentally including squamous sites in the analysis and the operators ability to simply repeat the measurement in real-time. The impact of falsely included squamous sites is discussed in section 7.3.2.

Finally, the AUC was only 0.95 in a prospective test. This may be a true limitation of the ESS system but there could be interpretation errors that might account for this loss of accuracy. These are extremely difficult to quantify without reanalysing approximately 700 biopsies but at the time that these samples were reported only significant areas of squamous mucosa were reported if present in a biopsy displaying mainly BO. In a personal communication from Professor Marco Novelli these Barrett's samples could have contained small areas (usually a few crypts) of squamous mucosa and this would often not have been reported and squamous mucosa in biopsies from BO has been demonstrated by other groups (Bridlestone et al, 1998). The interrogation

volume of the ESS probe is small compared to that of a biopsy for histology and in a large dataset it is likely that some readings were collected from one of these areas. Inaccurately labelled or mixed mucosal spectra could significantly affect the accuracy of any discriminatory algorithm produced.

### ***7.3.2 Elastic Scattering Spectroscopy for the detection of High Grade Dysplasia in Barrett's Oesophagus***

#### **Aim**

To determine whether ESS could detect HGD in Barrett's Oesophagus *in vivo* from matched optical measurements and biopsies collected for histology.

#### **Methods**

Matched ESS Spectra and biopsies for histology were collected using the methodology described above. The specimens collected for histology were reviewed by three independent histopathologists. Pathologist 1 reported the slides independently and the other two in consensus but blinded to pathologist 1's interpretation. In cases of discrepancy all three pathologists met to discuss a slide to see if an overall consensus could be reached. If this did not resolve disagreement then the matched site was not included in the final analysis. Furthermore, the pathologists were asked to report if HGD was focal (<50% of the surface of the biopsy involved) or widespread (>50% of the surface involved). Only widespread abnormalities were used for algorithm generation but both widespread and focal in the testing of the generated algorithm. Statistical analysis of the matched spectra was performed in the usual way (described in Chapter 6.5) for both jackknife and bootstrap analyses.

#### **Results**

In total 81 patients were recruited and 234 matched optical measurements were compared with histology. In the final analysis 181 sites were used corresponding to 595 spectra after 16 sites were excluded because their spectra had obvious artifacts following acquisition and in 37 cases the pathologists could not agree a histopathological diagnosis. Of the high risk sites (HGD or

intramucosal cancer) 12 biopsies (27%, 33 spectra) contained focal abnormalities and were therefore only included in the testing stage.

***Inter-observer variation of histological slides:*** The histological assessments of the columnar-lined biopsies are shown in table 7.1.

**Table 7.1: Distribution of the columnar-lined biopsy sites as assessed by the three pathologists (1 independent and 2 in consensus)**

	<b>Cambridge consensus diagnosis</b>	<b>UCLH independent diagnosis</b>	<b>Sites Agreed</b>	<b>Spectra available for Analysis</b>
<b>Vienna 5 (Cancer)</b>	12	14	<b>10</b>	<b>32</b>
<b>Vienna 4 (HGD)</b>	37	35	<b>35</b>	<b>103</b>
<b>Vienna 3 (LGD)</b>	28	16	<b>5</b>	<b>18</b>
<b>Vienna 2 (Indefinite for dysplasia)</b>	15	16	<b>2</b>	<b>8</b>
<b>Vienna 1 (no dysplasia)</b>	130	147	<b>129</b>	<b>434</b>
<b>Inadequate</b>	8	0		
<b>Total</b>	<b>218</b>	<b>218</b>	<b>181</b>	<b>595</b>

Kappa statistics were used to determine whether the agreement between UCLH and Cambridge pathologists was greater than expected by chance alone. This was found to be very much better for slides demonstrating cancer, high grade dysplasia and non-dysplastic Barrett's oesophagus than for low grade dysplasia or indefinite for dysplasia. The overall Kappa value suggested good agreement amongst these pathologists for the Vienna classification as a whole (Kappa=0.68, table 7.2).

Of much greater practical relevance is defining an individual's cancer risk. When patients were considered as either high or low risk (for ESS algorithm development) the pathologists demonstrated even better agreement for this classification (Kappa=0.72, CI 0.66-0.78).

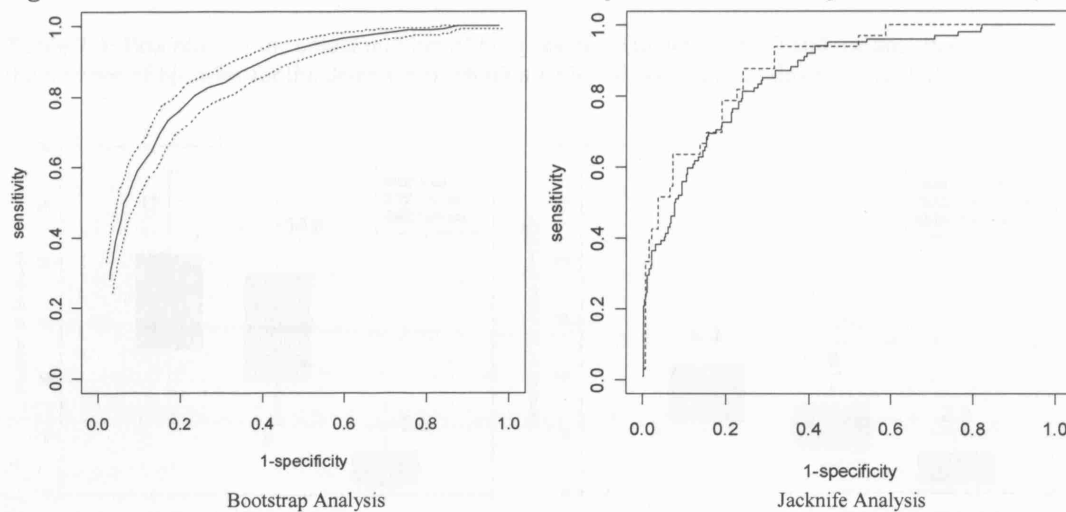
**Table 7.2: Summary of inter-agreement Kappa values for the comparison of pathologists in the analysis of oesophageal biopsies.**

	<b>Calculated Kappa</b>	<b>95% confidence interval</b>	<b>Strength of Agreement</b>
<b>Cancer (Vienna 5)</b>	0.76	0.74-0.79	Good
<b>High grade dysplasia (Vienna 4)</b>	0.66	0.61-0.70	Good
<b>Low grade dysplasia (Vienna 3)</b>	0.22	0.19-0.24	Fair
<b>Indefinite for dysplasia (Vienna 2)</b>	-0.02	-0.04-0.00	None
<b>BO without dysplasia (Vienna 1)</b>	0.61	0.53-0.69	Good
<b>Total</b>	0.68	0.61-0.74	Good
<b>Vienna 4 or 5 versus Vienna 1,2 or 3</b>	0.72	0.66-0.78	Good

**Accuracy of algorithm for the detection of HGD:** The results of the jackknife and bootstrap analyses for the detection of extensive HGD or cancer are shown below in figure 7.4. The bootstrap analysis produced a similar AUC of 0.82 (95% Confidence Interval 0.79-0.85) and the jackknife analysis an AUC of 0.85. The ‘cut-off’ canonical score between the groups was adjusted in order to load towards sensitivity maximising the algorithms chances for detecting HGD or cancer. This is important clinically as ‘missing’ a patient with HGD is potentially a far greater error than taking an additional biopsy. A sensitivity of 92% and specificity of 60% for the jackknife analysis were therefore chosen.

When the algorithm was applied to biopsies with focal HGD/cancer lying within otherwise normal mucosa, the sensitivity for detecting the abnormality fell only slightly to 85% with the specificity obviously remaining the same.

**Figure 7.4:** ROC Curve for ESS in the detection of widespread HGD, Bootstrap and Jackknife Analyses

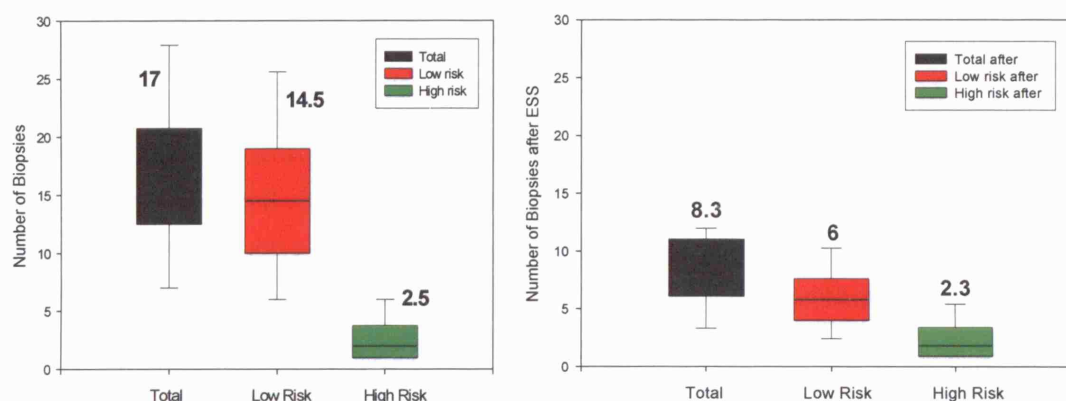


***Estimated number of biopsies required to detect HGD in a surveillance population:*** Once the sensitivity and specificity of ESS optical measurements have been calculated, it becomes possible to estimate of the number of biopsies required to detect HGD or cancer at surveillance endoscopy. This number is dependent on the prevalence of HGD and cancer in the surveillance population.

We reviewed the number of biopsies showing HGD or intramucosal cancer in a series of 20 patients who were referred to us for management. The average number of biopsies taken was 17 per endoscopy with a mean of 1.8 showing extensive HGD or cancer and a further 0.7 biopsies showing focal HGD or cancer.

Of crucial interest to a patient in this surveillance program is that high risk areas are not missed. There would be a tiny reduction in the number of sites of HGD or cancer detected but each patient has on average 2.5 sites containing HGD or cancer, so the chances of missing all of the sites are very much lower still (estimated sensitivity >99.7%). If this algorithm was translated to clinical practice a patient enrolling for surveillance endoscopy would no longer require quadrant biopsies every 2cm. Instead for example, 17 optical measurements could be taken and the number of biopsies necessary for histological examination would fall from a mean of 17 to 8 per endoscopy in those with HGD and 7 in those without. Figure 7.3 illustrates this possible reduction in the number of biopsies required with ESS guidance in those patients with HGD compared to biopsies taken without ESS guidance. A histopathologist would on average look at 60% less 'low-risk' biopsies. Furthermore, a negative standard surveillance endoscopy performed with the assistance of ESS would have a negative predictive value in excess of 99.5%.

**Table 7.3:** Box plots of the actual number of biopsies taken to detect HGD and the anticipated reduction in the number of biopsies for the detection of HGD if ESS had been used in this High Risk Population



**False positive rate for HGD amongst squamous sites:** The spectra from 100 squamous sites were analysed using the algorithm generated above. Of these 100 sites 63 were incorrectly diagnosed as HGD. This potentially represents a serious problem because if the endoscopist inadvertently collects one or more readings from a squamous area there is a high likelihood of a false positive result.

## Discussion

ESS appears to be able to detect HGD and intramucosal cancer in BO. If the optical measurement suggests the absence of high grade dysplasia or cancer throughout an entire Barrett's segment then the estimated negative predictive value in a surveillance population is well in excess of 99.5%. The specificity in this study suggests that ESS will also decrease the number of low risk biopsies the pathologist reviews by 60% (specificity) and be associated with almost no loss in accuracy.

The accuracy of our model is based on our finding that 'on average' a patient at high risk will have 2.5/17 biopsies with HGD when quadrant samples are taken every 2cm. This is consistent with Cameron's finding that, when present, high-grade dysplasia and cancer cover an average of 10% of the Barrett's oesophageal mucosa (Cameron and Carpenter 1997). Since optical spectra are easy and quick to collect clinicians might wish to take measurements every 1 cm instead of

every 2 cm leading to a further increase in accuracy of diagnosing abnormalities (Reid et al. 2000a). As the probe does not need to be removed unless a biopsy is needed for histological examination this makes it possible to survey a Barrett's segment without dysplasia quickly. Proof of these concepts of collecting optical samples every 1cm and the speed of collecting these measurements will be assessed further in the study in part 7.2.4 below.

The ability of ESS to differentiate squamous from Barrett's mucosa becomes more important. This is due to the fact that if measurements are inadvertently collected from squamous mucosa they cause a false positive rate for HGD in 63% of cases if inputted into the HGD detection algorithm. The accuracy of the discrimination between squamous mucosa and Barrett's Oesophagus described above is good (AUC 0.95) potentially allowing inadvertently collected squamous spectra to be automatically removed prior to the analysis for HGD. It is unlikely that these squamous spectra would be collected with long segment circumferential Barrett's but in 'patchy' non circumferential Barrett's with or without islands more commonly found in a surveillance population this could result in significant difficulty for the endoscopist. An automated solution would be far superior to careful measurement collection which would not be ideal for translation into routine clinical practice.

There are, however, limitations to this study. First is the difficulty in accurately diagnosing HGD using the gold standard of pathology. Histopathologists find it difficult to agree consistently about the diagnosis of HGD. It is difficult to suggest under these circumstances that ESS should be completely accurate as it was necessary to use pathologist consensus to train our algorithm. Secondly, the number of patients in this dataset is small (81 patients, with 181 biopsy sites of which 45 were high risk) and a far larger training set would allow more confidence in the results. Perhaps most importantly, a prospective test of this algorithm is required to ensure the validity of these results as well as testing the device in a surveillance examination taking quadrant measurements every one or two centimetres.



### ***7.3.3 Elastic Scattering Spectroscopy to generate an image of patient risk in Barrett's Oesophagus.***

#### **Introduction:**

ESS is a point measurement but readings can be collected extremely quickly (<200ms per spectra) offering clinicians the opportunity to rapidly take multiple readings from an area of Barrett's mucosa. One of the principle weaknesses of ESS as a technology is that it is a point measurement and is therefore reliant on being in contact with the abnormal mucosa prior to the optical measurements being taken. Additionally, the only studies of ESS to date used matched optical measurements to histological biopsies with only a few readings collected from each patient. These readings were typically targeted to high risk patients in order to obtain enough abnormal sites (HGD or aneuploidy) for algorithm development. No studies have used ESS to try to survey an individual patient with Barrett's Oesophagus offering a diagnosis as to whether these *individuals* are at high or low risk. Ultimately for use in surveillance patients the ESS algorithm needs to be able to collect many readings rapidly from these patients (at least quadrant spectra every 2cm of BO) and be robust enough to be used by multiple users on busy endoscopy lists without any specific periods of extended training.

Making the assumption that any optical readings collected from adjacent sites (eg posterior and left quadrants of BO at the same level or posterior quadrants one cm apart) are not entirely independent it may be possible to linearly interpolate the canonical score results from the ESS statistical algorithm from adjacent sites to form a virtual 'image' of the oesophagus. Linear interpolation is simply a method for filling gaps in any table or graph and refers to graduating, in this case the canonical score, between two points with known values in linear and regular increments. This may not be unreasonable in the model of Barrett's Oesophagus because of the potential presence of fields of genetic and histological abnormalities (Cameron and Carpenter 1997; Maley et al. 2004) and because potentially the larger the value of the canonical score the greater positive predictive value for that site. A risk score, calculated by linear interpolation, could then be assigned to each point between these two sites. As multiple measurements are made these results could be formed into 'an image' of a patient's BO with a false colour map overlaid to represent the positive predictive value of an optical measurement within the Barrett's segment.

**Aims:**

- To prospectively test our previously generated algorithm for the detection of HGD.
- To test whether new spectra examined using this algorithm developed for the detection of HGD could also detect aneuploidy.
- To determine if the absolute value of the canonical score provided additional information with regard to the positive predictive value of that site for both HGD and aneuploidy.
- To determine, in a proof of concept study, if the algorithm predicted the overall patient risk for the presence of both HGD and aneuploidy following the generation of a scanned image of that patient's Barrett's Oesophagus. Particular reference was given to:
  - Was a specificity of 60% maintained for sites without HGD?
  - Were cases of HGD and aneuploidy 'missed' with entirely negative scans?
  - Was taking quadrantic optical biopsies every 1cm practical particularly with regard to procedure time?
  - Was there significant inter-operator variability?

**Methods:**

A flow chart of the ESS datasets used in this study is shown in figure 7.5 below.

Matched biopsies were taken from patients in a prospective test to examine the accuracy of the previously developed algorithm for the detection of HGD defined in section 7.3.2. The dataset used to produce the results described in section 7.3.2 above used as the training set and new completely independent data formed a simple prospective test set for the diagnosis of HGD. An independent prospective test set for the diagnosis of aneuploidy was also used to examine whether this algorithm developed for HGD could also detect aneuploidy. The diagnosis of aneuploidy was made using image cytometry with all histograms reported by two blinded observers who met to agree a consensus in cases of disagreement.

The algorithm for spectral statistical analysis produces results in the format of a continuous variable the 'canonical score'. Rather than just place a binary positive or negative outcome on this score its absolute value may hold additional information. The actual canonical score might relate to the risk of a site showing HGD or aneuploidy (ie the higher the score the higher the risk). The

results of the prospective test would be examined to determine whether the size of the canonical score really related to the PPV of those sites.

Four different operators were involved in the collection of ESS readings, two of whom were entirely unfamiliar with the technology. These two ‘new’ operators were given a five minute introduction to the technology, demonstrated its use and then allowed to collect data for the study. Four ESS measurements were taken in rapid succession from each site and quadrantic sites collected every 1cm of Barrett’s Oesophagus. The optical image of a patient’s Barrett’s oesophagus was then created by interpolating the statistical scores between optically measured sites and then a false colour map was laid over the top to express the positive predictive value at each site. These images were then analysed against quadrantic biopsies taken every 2cm of Barrett’s. These biopsies were first processed for histology to look for the diagnosis of HGD. A biopsy without HGD was further processed for aneuploidy using image cytometry in the standard way described in Chapter 5.

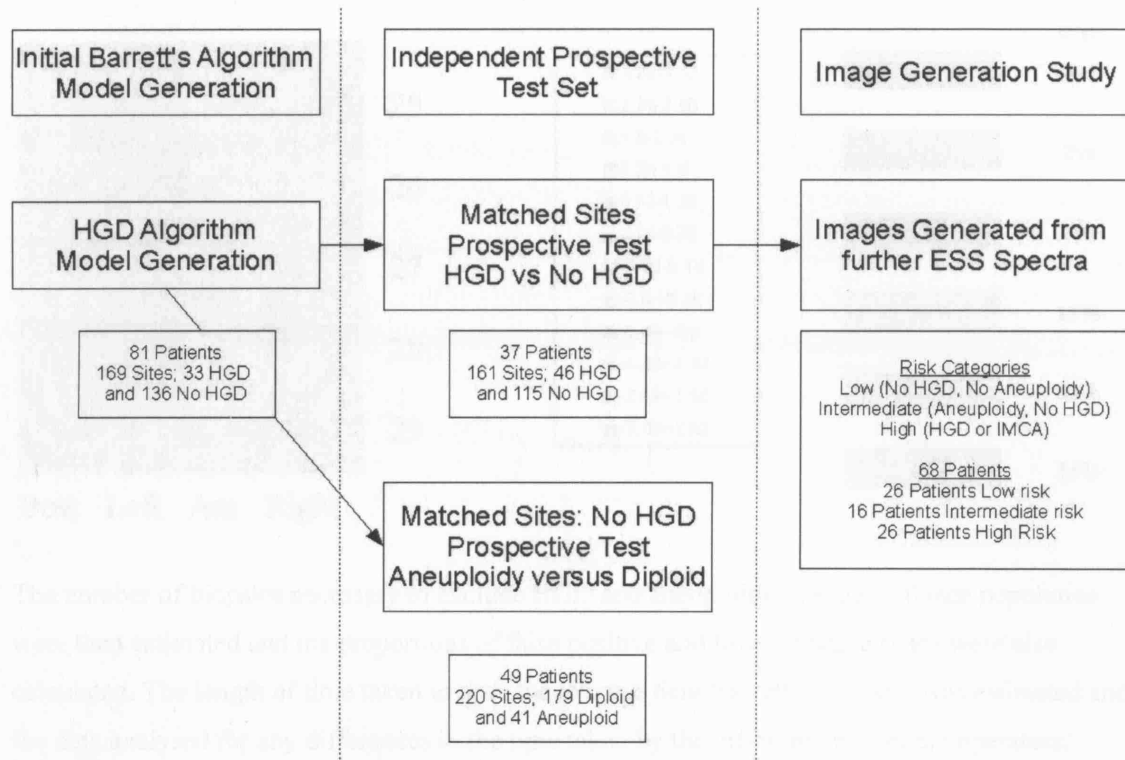
The patients were then assigned to three risk categories depending on whether HGD or Aneuploidy was found in their biopsies. The three risk categories were low, intermediate or high risk. These risk categories, the criteria for inclusion into that group and the estimated 5 year cancer risk of those groups based on the Seattle dataset are shown in table 7.4 below.

**Table 7.4:** Risk Categories and their criteria for ESS Image Study

<b>Risk Category Assigned</b>	<b>Criteria for inclusion</b>	<b>5 year cancer risk*</b>
Low	All biopsies without HGD and diploid on ICM	0%
Intermediate	No HGD, Aneuploidy on ICM	28%
High	HGD or IMCA on histology	45-65%

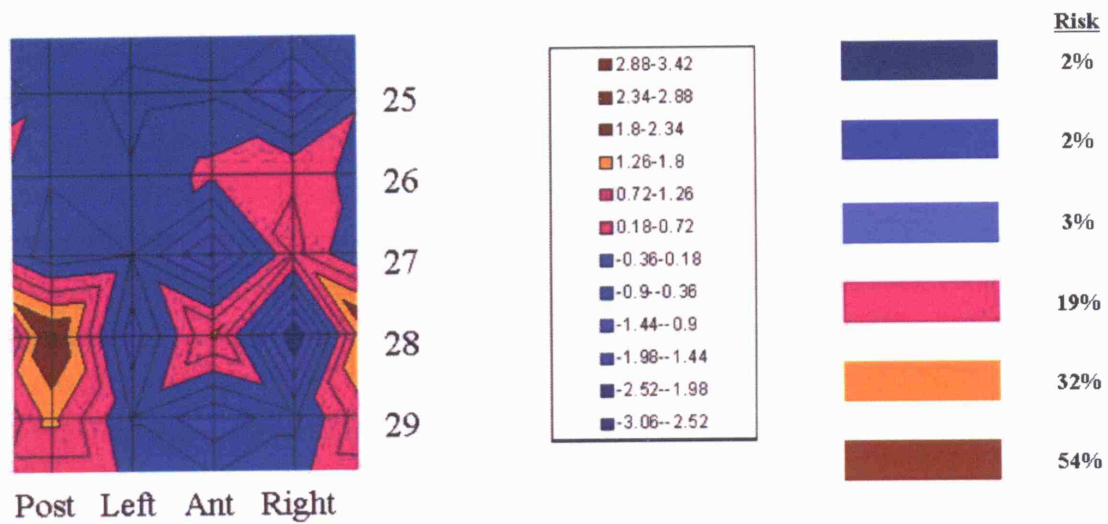
\*(Reid et al. 2000b)

**Figure 7.5: ESS Datasets their size and use in this study**



In the example of a Barrett's image (figure 7.6 below), the cylindrical shape of the oesophagus was flattened out by an imagined incision between the right and posterior quadrants. The distance of the optical measurement from the incisors was printed on the right hand side of the image in centimetres and the quadrant position (posterior, left, anterior, right) printed at the bottom. Each canonical score is then plotted at its respective position and the scores interpolated to fill in the 'gaps' between points on the image on the left. The canonical scores were then assigned a colour, defined by the legend in the middle box, which was then laid over the actual score on the image on the left to produce the false colour map of risk. On the right there is a simplified schematic showing each colour and the PPV or risk of that site harbouring HGD or aneuploidy. The PPV is explored in more detail in the results section below.

Figure 7.6: Example of ESS Scan



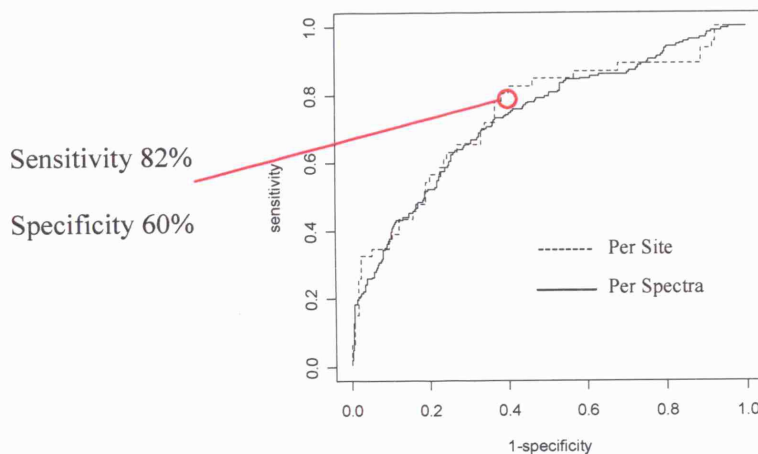
The number of biopsies necessary to exclude HGD and aneuploidy in a surveillance population were then estimated and the proportions of false positive and false negative scans were also calculated. The length of time taken to scan the average 6cm Barrett's segment was estimated and the data analysed for any differences in the time taken by the different endoscopic operators.

## Results:

### *Prospective Testing for HGD*

A prospective test of matched sites of HGD versus LGD or no dysplasia (no HGD) using the algorithm developed in section 7.3.2 was performed on 37 patients with 645 spectra collected from 161 sites of which 46 showed HGD and 115 sites without HGD. The area under the ROC curve was 0.74 displaying reasonable accuracy albeit less than in the original algorithm development stage (AUC 0.85). The dotted line is a per spectra accuracy and the solid line a per site accuracy; a sensitivity of 82% and specificity of 60% could be chosen from this curve (circled). See Figure 7.7.

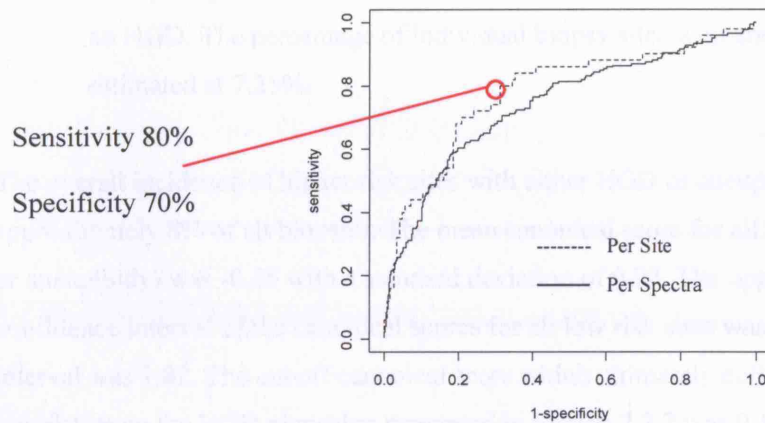
**Figure 7.7:** Matched results for HGD in a Prospective Test



### *Prospective Testing for Aneuploidy*

When the HGD algorithm (section 7.3.2) was prospectively tested on an independent dataset of sites with and without aneuploidy in the absence of HGD it was accurate at predicting aneuploidy. Forty-nine patients had ESS spectra collected from 220 sites after those sites displaying HGD were removed. One hundred and seventy-nine diploid sites and 41 sites with image abnormalities were examined. Agreement for the reporting of aneuploidy from histograms generated using image cytometry was reached by both observers in all cases. The area under this ROC curve for the detection of aneuploidy compared to sites without HGD or aneuploidy was 0.78. See Figure 7.8. The results below suggested a possible cut off for aneuploidy using the HGD algorithm produced a sensitivity of 80% and specificity of 70% (circled). Although expected, this suggested that this algorithm had a high false positive rate for aneuploidy probably because approximately two thirds of biopsies with HGD also have aneuploidy.

**Figure 7.8:** On sites without HGD the statistical algorithm detected aneuploidy in a prospective test



The data suggested that when an image is produced from the ESS measurements analysed using the pre-defined HGD algorithm it may not only distinguish patients with HGD but also those with aneuploidy.

*The canonical score in the estimation of the positive predictive value*

Additionally, data from the absolute value of the canonical score was shown to be useful in defining the PPV. As the canonical score increased the lower the likelihood of a false positive result became and the greater the probability that the site tested would display HGD or aneuploidy on biopsy.

The real variable of interest to a clinician is the positive predictive value (PPV) of a site being positive for either HGD or aneuploidy given a positive ESS result in a surveillance population. In order to calculate the PPV for each site an estimation of the relative frequencies of HGD and aneuploidy in this surveillance population was necessary.

- The incidence of HGD in BO has been suggested as approximately 1.3%/year. Assuming one endoscopy every 2 years the incidence per surveillance endoscopy is 2.6% (Sharma et al. 2006).
- HGD has been shown to be only present in 2.5 out of 17 biopsies on average in those patients with HGD (Lovat et al. 2006). The percentage, therefore, of biopsy sites with HGD in a surveillance population can be estimated as 0.38%.



- The prevalence of aneuploidy in a surveillance population was estimated to be 13% in the Seattle groups cohort of patients without HGD (Reid et al. 2000b).
- Aneuploidy was present in 29/52 samples in this study of 16 patients with aneuploidy but no HGD. The percentage of individual biopsy sites with aneuploidy was, therefore, estimated at 7.25%.

The overall incidence of higher risk sites with either HGD or aneuploidy was estimated as approximately 8% of all biopsies. The mean canonical score for all low risk sites (without HGD or aneuploidy) was -0.55 with a standard deviation of 0.92. The upper limit of the 95% confidence interval of the canonical scores for all low risk sites was 1.25 and the 99% confidence interval was 1.82. The cut off canonical score which ultimately defined if a site was either high or low risk from the HGD algorithm generated in section 7.3.2 was 0.18. This cut-off was therefore used in this prospective test. The results of the calculations for PPV are presented in table 7.5 below. The statistical output, the canonical score, is in the left column, the risk of that site having a positive diagnosis of HGD or aneuploidy in the middle column and the colour assigned to that risk is presented in the right column.

**Table 7.5:** Positive predictive values dependent on canonical score

Canonical Score	PPV HGD & Aneuploidy	Colour Assigned on ESS Maps
>1.82	0.54	Purple
>1.25	0.32	Orange
>0.18	0.19	Pink
<0.18	0.03	Light Blue
<-0.92	0.02	Blue
<-1.92	0.02	Dark Blue

#### *Image Generation of a patient's Barrett's Oesophagus using ESS*

Sixty-eight patients had their BO scanned using the methodology described above. Twenty-six had HGD on subsequent biopsies and 42 had either low grade dysplasia or no dysplasia. Of these 42 patients without HGD, 16 were found to have aneuploidy and 26 were diploid.

Table 7.6 shows the mean length of BO and the range between the different risk groups. When compared to the low risk group there was no difference in Barrett's length between this group and the intermediate risk group but there was a non-statistically significant trend towards increased Barrett's length for HGD.



**Table 7.6:** Length of Barrett's Oesophagus in different risk groups of patients having ESS Image created

Patient Risk	n	Length of BO (Mean, Range)	t test p-value
Low (No Aneuploidy, No HGD)	26	4.5cm, 1-12cm	Comparator
Intermediate (Aneuploidy, No HGD)	16	5 cm, 2-7cm	0.36
High (HGD)	26	6cm, 1-12cm	0.07

A positive scan was defined as any scan with one or more positive sites (ie with a canonical score greater than or equal to 0.18) and these sites would appear pink, red or brown on the ESS scan.

All 26 patients with HGD and all 16 with aneuploidy had positive scans and all these patients had a positive area of the scan approximating to the position of HGD or aneuploidy found on subsequent biopsy. See figures 7.9 and 7.10. Seven out of twenty-six (27%) low risk patients with no dysplasia and no aneuploidy had entirely negative scans. If biopsies were directed only at suspicious areas on the scans in these 26 patients' without HGD or aneuploidy 106 instead of 258 biopsies (41%) would have been required to exclude HGD. Furthermore, when examining a posterior series for aneuploidy in order to more accurately determine these patients five year risk of cancer development if biopsies were again targeted to sites with a positive ESS spectra only 23 instead of 61 (38%) would have been necessary to exclude aneuploidy. See figure 7.11. If both HGD and aneuploidy could be excluded then routine surveillance could reasonably be deferred for at least 5 years. Scanning the oesophagus optically was performed by four different operators and took an average of 4 minutes and 22 seconds for a 6cm segment of Barrett's, with no significant difference between the operators.

**Figure 7.9:** Example of High Risk ESS Scan

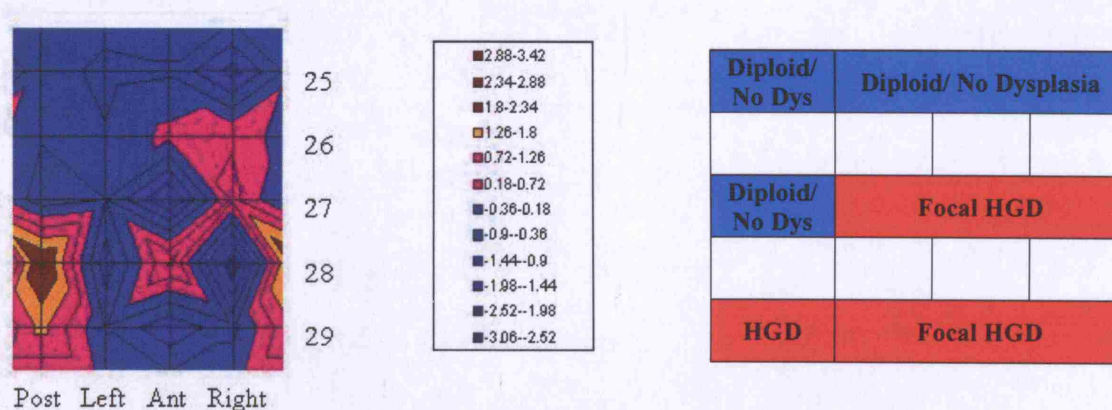


Figure 7.10: ESS Scan of patient with Aneuploidy

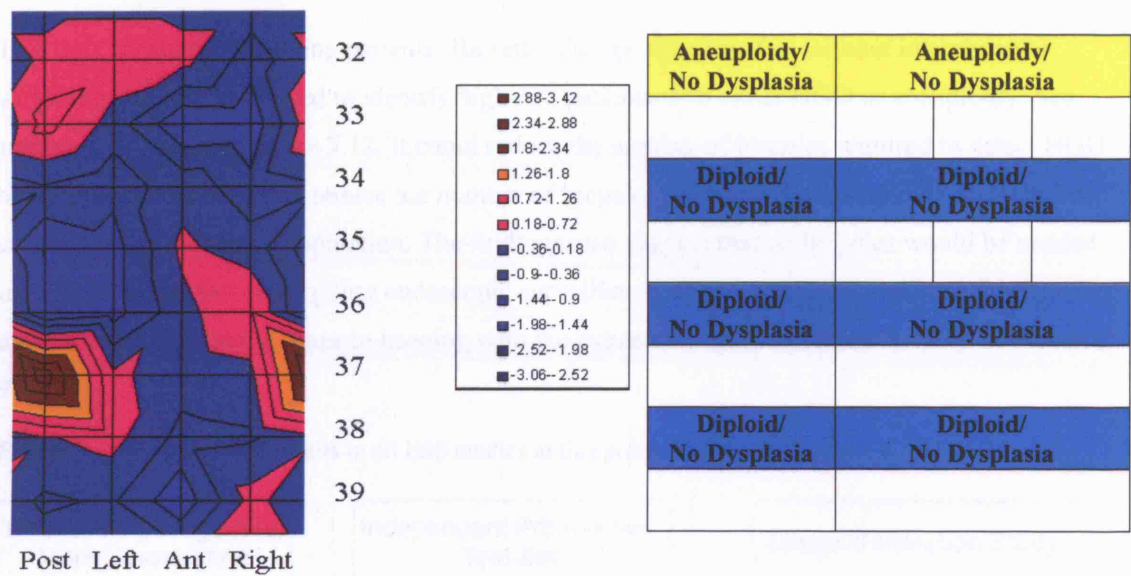
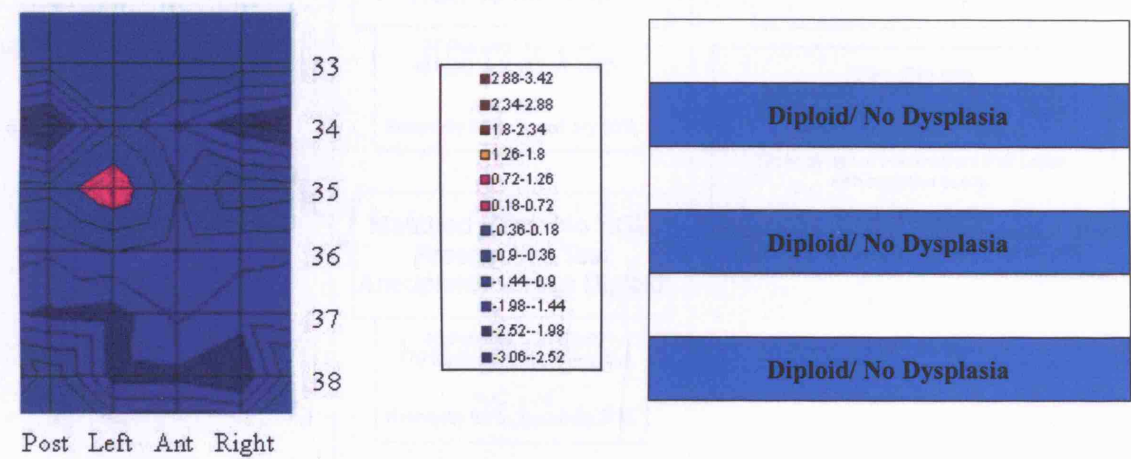


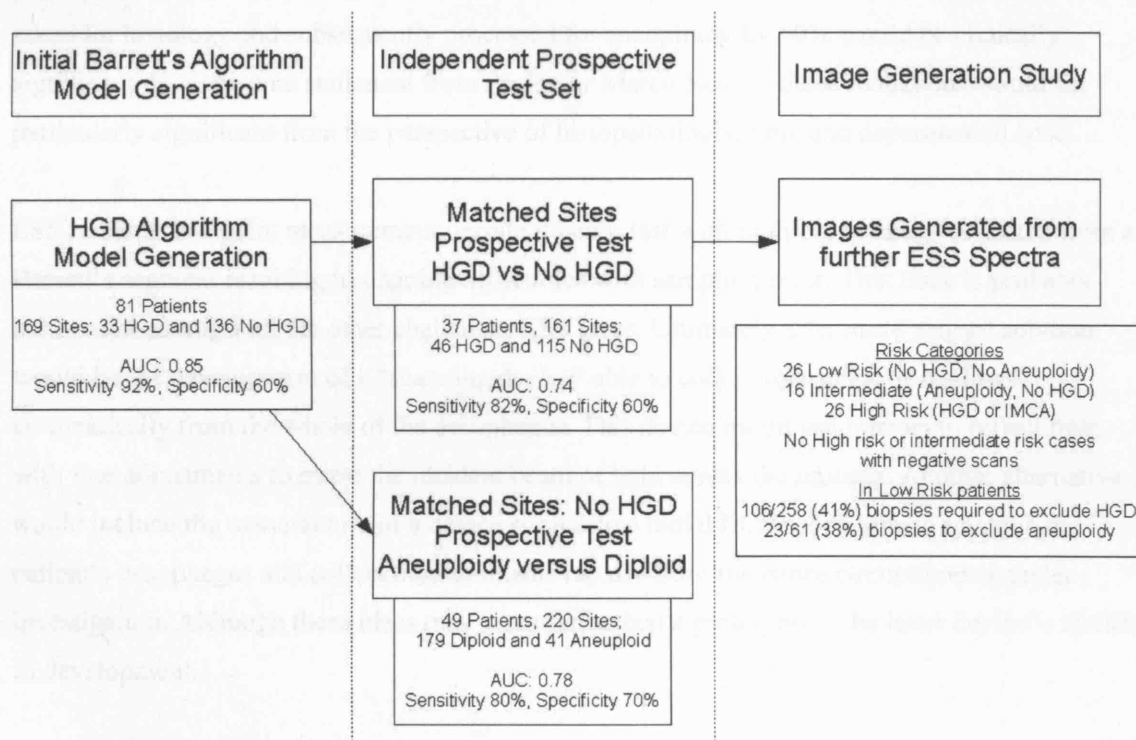
Figure 7.11: Example of Low Risk ESS Scan



## Conclusions:

This data shows that scanning patients' Barrett's Oesophagus was fast without interobserver variability and could be used to identify high risk patients with either HGD or aneuploidy. See summary of results in figure 7.12. It could reduce the number of biopsies required to detect HGD by approximately 60% and reduce the number of biopsies processed for aneuploidy by a similar amount in a surveillance population. The findings also suggest that no biopsies would be needed in 27% of all patients undergoing endoscopic surveillance. In this series, no patients with HGD or aneuploidy had negative scans in keeping with the expected negative predictive value in excess of 99.5%.

**Figure 7.12:** Summary of results in all ESS studies at this point



There are serious limitations associated with this study. These primarily surround the fact that the software at the time of the study did not allow for 'online real-time' results within the endoscopy room. This prevented the true targeting of biopsies to high risk sites for subsequent analysis which resulted in comparison with just quadrantic biopsies every 2cm. This means that a patient's diagnosis overall was considered with the proximity of quadrantic biopsies every 2cm showing aneuploidy and HGD compared to the optical measurements. It was not possible, however, to

truly present the accuracy of ESS with certainty surrounding the proportion of biopsies saved without targeted biopsies.

A second limitation is that the above data did not include a dedicated algorithm for the detection of aneuploidy. This study does however raise the possibility of ESS being able to detect aneuploidy in vivo in the absence of HGD and this is studied in section 7.3.4 below.

The study, however, did fulfil the aims stated. It appeared to suggest that sensitivity remained excellent with an extremely high negative predictive value but the specificity remained at approximately 60%. It also seemed that there are no significant inter-operator differences and that a Barrett's segment could be surveyed in a short period of time taking four optical measurements per site, quadrantic sites every cm of BO. The final question was whether a reduction in biopsies taken for histology and subsequently processed for aneuploidy by 60% would be clinically significant. In a personal statement from Professor Marco Novelli these reductions would be particularly significant from the perspective of histopathologist time and departmental costs.

ESS remains as a point measurement despite it being fast with multiple readings collected from a Barrett's segment resulting in ongoing difficulties with sampling error. This issue is probably paramount amongst all the other challenges ESS faces. Ultimately a far more elegant solution would be the development of a "scanning device" able to collect thousands of readings automatically from the whole of the oesophagus. This device might use mirrors to reflect light with fine adjustments to move the incident beam of light across the mucosa. Another alternative would include the manufacture of a device similar to a radial EUS scope able to rotate in the patient's oesophagus and collect measurements rapidly from the entire circumference under investigation. Although these ideas may seem farfetched a prototype of the latter device is already in development.

Despite the limitations of this proof-of-concept study, it does support prospective testing with image generation and targeting of biopsies using ESS in the endoscopy room for the detection of both HGD and aneuploidy in BO.

#### ***7.3.4 Elastic Scattering Spectroscopy for the detection of Aneuploidy in Barrett's Oesophagus***

##### **Introduction:**

For scientific validity the entire dataset was used initially to assess whether ESS could detect aneuploidy although as shown above there is a false positive rate between HGD and aneuploidy. In the absence of a multivariate four-way classification if HGD is included in the analysis, it must be accepted as a potentially confounding variable. This model generation using the entire dataset irrespective of dysplasia and its prospective testing is shown in section 7.3.4.1. A more robust and potentially clinically useful analysis however would be model generation and testing on a subgroup of biopsy sites without HGD. The real clinical question, particularly in light of the data presented above, is whether ESS is able to detect aneuploidy in the absence of HGD. This is examined in section 7.3.4.2.

##### **Aims:**

- To assess whether ESS could detect aneuploidy *in vivo* in Barrett's Oesophagus
- To assess whether ESS could detect aneuploidy *in vivo* in BO in the more clinically relevant dataset of biopsies that did not contain HGD.

#### ***7.3.4.1 Elastic Scattering Spectroscopy for the detection of Aneuploidy in Barrett's Oesophagus***

##### **Methods:**

Matched optical and conventional biopsies were taken from patients with Barrett's oesophagus and biopsies were collected in two independent datasets; one for algorithm development and one for algorithm testing.

In the dataset for algorithm development all 169 sites in the previously developed HGD algorithm in section 7.3.2 were processed for aneuploidy and tetraploidy using image cytometry by the method described in section 5.3. All histograms produced for the assessment of aneuploidy were reported blindly by two observers (Prof M Novelli and Dr G Mackenzie) using the criteria defined in section 5.6. These observers met to agree a consensus in cases of disagreement. Two statistical analyses were performed; a block validation statistical 'bootstrap' analysis and a leave one out cross validation statistical 'jackknife' analysis.

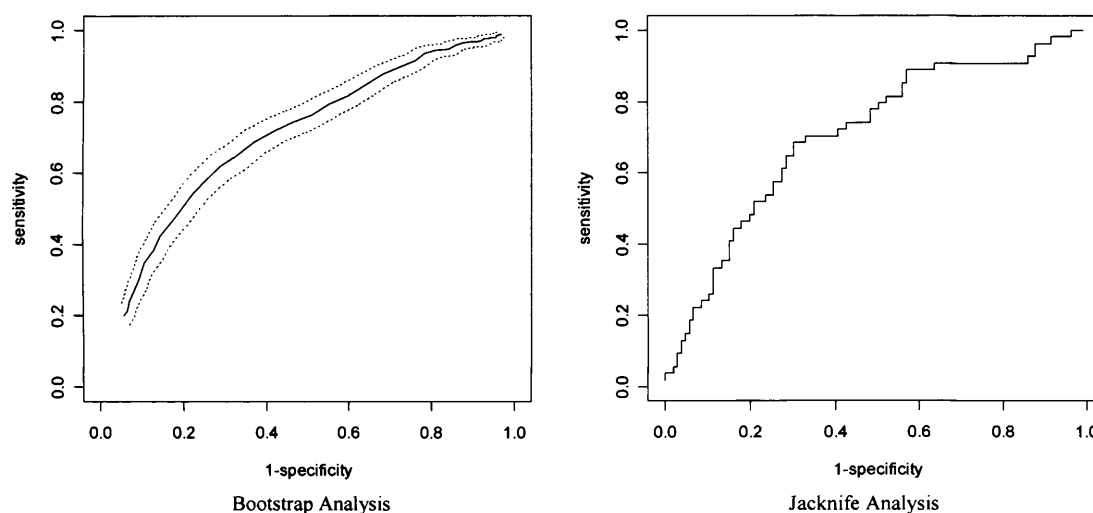
The algorithm developed in this model generation step for the detection of aneuploidy all for sites irrespective of their histological findings was then prospectively tested in an independent test set.

## Results:

Histograms were blindly analysed by two observers and consensus was reached in all cases (Prof M Novelli and Dr G Mackenzie).

In the algorithm development step 81 patients with 160 sites completed the analysis for image abnormalities; 106 were diploid and 54 displayed ICM abnormalities. Nine biopsies (without HGD) did not contain enough residual tissue in the biopsy blocks to take a further 40 micrometre section and due to issues of tissue preservation for clinical purposes these samples were not processed. In total this dataset contained 33 biopsies displaying HGD. In this algorithm development step both 'bootstrap' and 'jackknife' analyses demonstrated an AUC of 0.71 and a sensitivity and specificity of 70%. See figure 7.13.

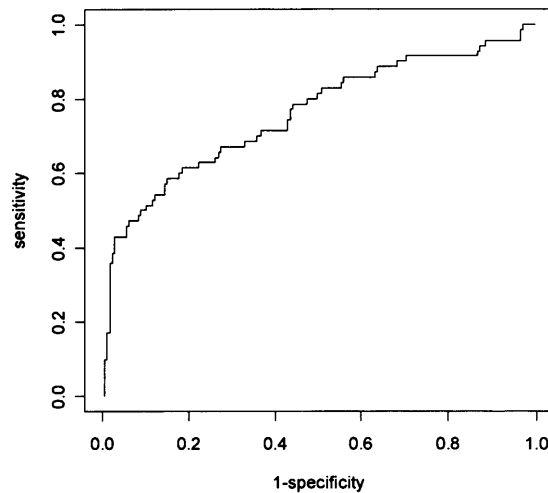
**Figure 7.13:** ROC curve of ESS results for the detection of aneuploidy in algorithm generation with all sites included in the analysis irrespective of grade of dysplasia



This algorithm was then prospectively tested in an independent test set collected from 49 patients consisting of matched optical measurements with histological biopsies. Of these 249 biopsies, 179 were diploid and 70 displayed aneuploidy or tetraploidy. This dataset excluded 4 biopsy

blocks that did not contain enough residual tissue for image analysis. This prospective test demonstrated an AUC of 0.76 and a sensitivity of 62% and specificity of 82%.

**Figure 7.14:** Prospective test of algorithm to detect aneuploidy developed using the algorithm generated on all sites in figure 7.13



#### **Discussion:**

These results were encouraging that ESS might be able to detect aneuploidy but HGD is a potential confounding variable in this univariate analysis. There are two potential solutions; the first is to exclude all sites with HGD in order to develop an algorithm purely for the detection of aneuploidy in sites without HGD. The results of section 7.3.2 have shown that ESS can detect HGD reliably and section 7.3.3 has shown the addition of a second algorithm to detect aneuploidy might not only allow the diagnosis of these patients but might also improve overall accuracy. The second solution, the development of a four-way multivariate classification, would be complex and time consuming to perform and evidence would be required to justify this investment of time and effort. Section 7.3.4.2 examined the plausibility of ESS to detect aneuploidy in the absence of HGD in a simple univariate analysis.

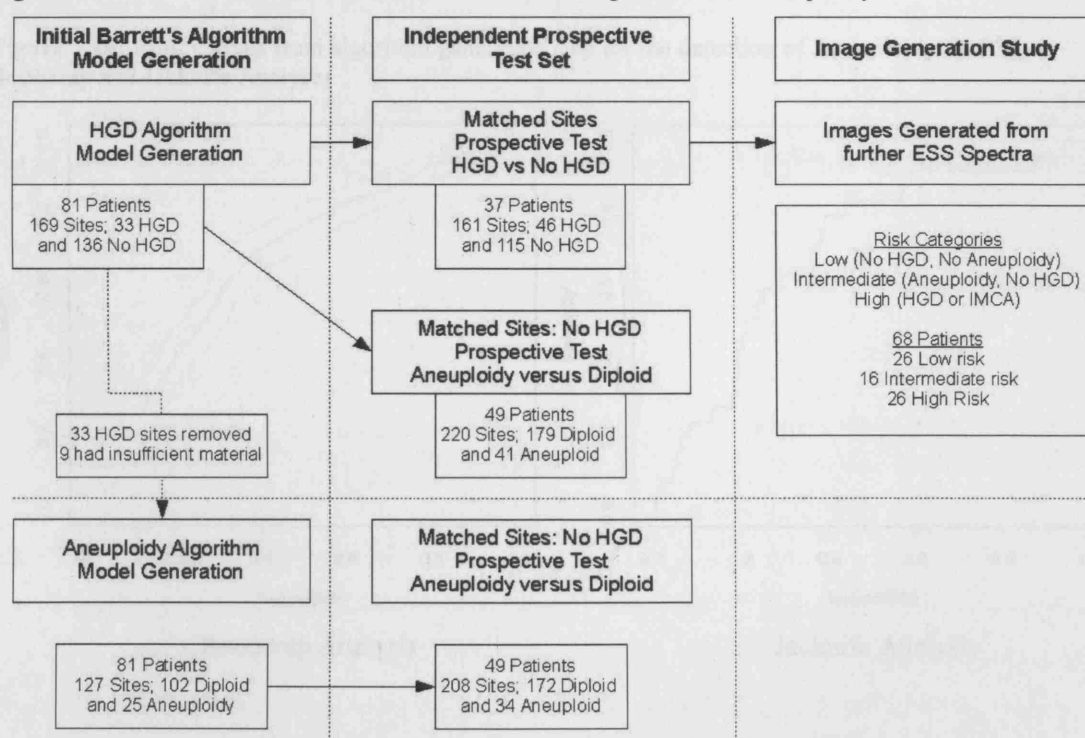


### 7.3.4.2 Elastic Scattering Spectroscopy for the detection of Aneuploidy in Barrett's Oesophagus in the absence of HGD

#### Methods:

The study performed in 7.3.4.1 was re-analysed with biopsies demonstrating HGD excluded. Section 7.3.2 showed that ESS can detect HGD with an AUC of 0.85 and section 7.3.3 demonstrated that the HGD algorithm was positive in many cases of aneuploidy (AUC 0.78). HGD is likely to be a confounding variable in any univariate analysis. One reasonable criticism of the study above is without multivariate analysis it may be that the ESS algorithm is still mainly detecting HGD. These sites containing HGD were therefore removed. Additionally, from the clinical perspective an optical technique that could detect a biomarker of future cancer risk in the absence of other abnormalities (ie HGD) would be a major advance. Three statistical analyses were performed. Firstly, an algorithm was developed using the initial dataset which was followed by a simple prospective test of this algorithm on the second independent dataset collected. Finally, an overall algorithm was generated using all the data available. The increased size of the dataset in this final algorithm should improve its overall reliability. See figure 7.15 below.

**Figure 7.15:** Flow Chart of all ESS studies to data including datasets for aneuploidy



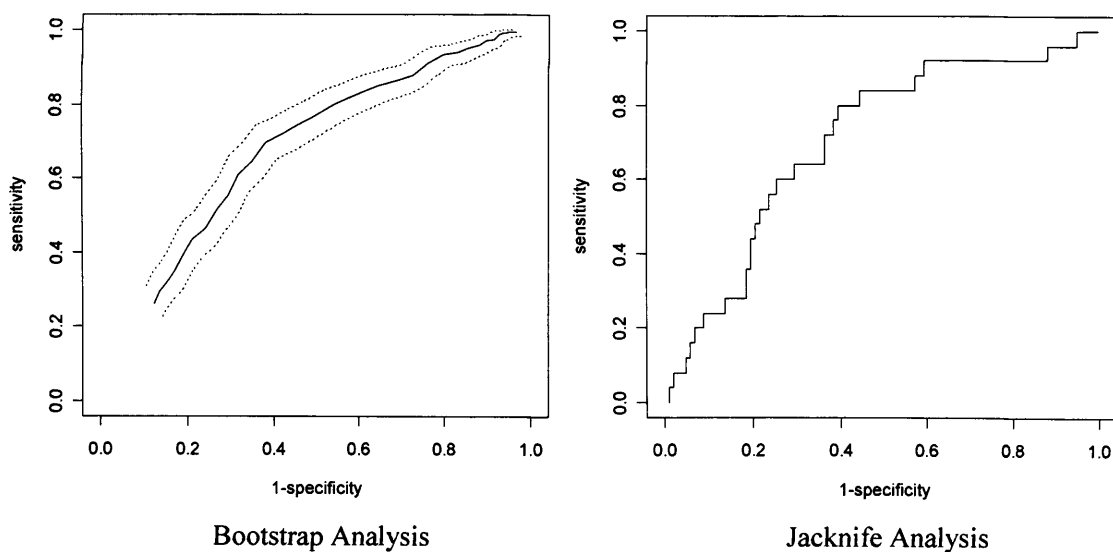


## Results:

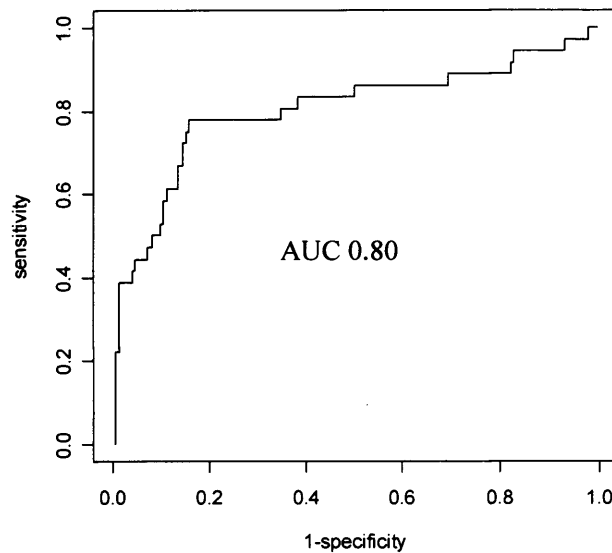
The dataset used in the development of the HGD algorithm above was taken and those sites without HGD were processed for aneuploidy and used as the training set. This training set consisted of 81 patients with 127 biopsies processed for aneuploidy of which 102 were diploid and 25 aneuploid. As discussed in section 7.3.4.1, 9 biopsies without enough residual tissue were not processed. The independent prospective test set consisted of 49 patients with 207 biopsies without HGD of which 171 were diploid and 36 displayed aneuploidy. Histograms were blindly analysed by two observers and consensus was reached in all cases. No sites contained HGD. A statistical algorithm using both jackknife and bootstrap training techniques was constructed to discriminate between aneuploid and diploid sites without HGD.

The results were almost identical using both statistical techniques. ESS correctly identified aneuploid sites using both statistical methods in the algorithm generation step with an AUC of 0.71. Figure 7.16. In the prospective test the area under the ROC curve was 0.80 displaying good discrimination. Figure 7.17. Overall the accuracy of ESS for the detection of Aneuploidy in the absence of HGD was reasonable with an AUC of 0.78. Figure 7.18.

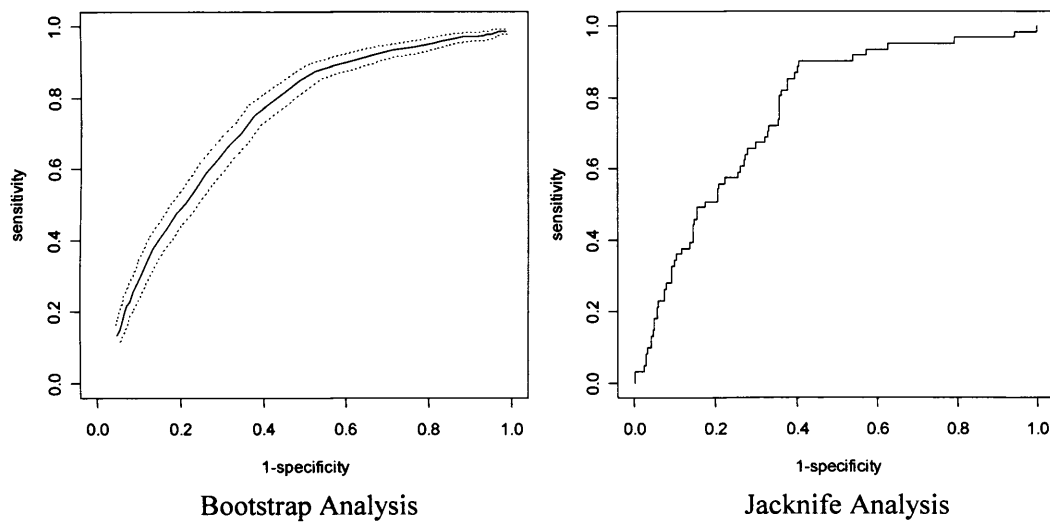
**Figure 7.16:** ROC Curves from algorithm generation step for the detection of Aneuploidy by ESS, Bootstrap and Jackknife Analyses



**Figure 7.17:** ROC curves for prospective test of above algorithm for the detection of Aneuploidy by ESS in Barrett's Oesophagus.



**Figure 7.18:** ROC curve of all data used in algorithm generation for the detection of Aneuploidy by ESS in Barrett's Oesophagus, Bootstrap and Jackknife Analyses



From the simple prospective test in figure 7.17 above an estimated sensitivity of 79% and specificity of 82% could be obtained from this dataset for the detection of aneuploidy in the absence of HGD. These results were chosen because they represent a more balance false positive and false negative rates than for HGD. This would seem preferable in the diagnosis of aneuploidy because missing the very occasional case of aneuploidy, which is not currently screened for is much more acceptable than missing a case of HGD.

In a small study, performed within the surveillance population at UCH, 16 patients without HGD were found to have aneuploidy or tetraploidy. From these 16 patients, 27/52 biopsies taken from posterior sites every 2cm of BO displayed aneuploidy using ICM. The mean number of biopsies processed per patient was therefore 3.2. Assuming this group of patients is representative of a general surveillance population in the UK without HGD and that 13% of a surveillance population have aneuploidy but no HGD (Reid et al. 2000b) then an estimate of the number of biopsies saved in aneuploidy processing and the positive (PPV) and negative predictive values (NPV) can be calculated.

The estimated number of biopsies that would no longer need to be processed to exclude aneuploidy from diploid patients is reduced by 82%.

The negative predictive value of an entirely negative posterior series of biopsies can be estimated to be:

$$\text{The chance of missing all sites of aneuploidy in a positive patient} = (1 - \text{Sensitivity})^{Sa}$$

where ***Sa*** is the average number of aneuploidy sites per patient

The percentage chance of missing a patient with aneuploidy in a surveillance population is therefore:

$$100 * (Pa((1 - \text{Sensitivity})^{Sa}))$$

Where ***Pa*** is the prevalence of aneuploidy in a surveillance population

Therefore, the negative predictive value (NPV) of a negative posterior series is:

$$100 - (100 * (0.13 * (0.21^{1.7}))) = 99.1\%.$$

The positive predictive value of any particular site can be calculated as follows:

Number of positive biopsies collected from patients with aneuploidy (***Aa***):

$$Aa = (Sa * Pa * \text{Sensitivity})$$

Number of negative biopsies taken from patients with aneuploidy (***Ad***):

$$Ad = ((1 - \text{Specificity} * Sd * Pa))$$

Where, ***Sd*** is the average number of diploid sites per patient

Number of negative biopsies taken from diploid ( $Dd$ )

$$Dd = ((1 - Specificity) * Sd * Pd)$$

where  $Pd$  is the prevalence of diploid patients in a surveillance population

The PPV is the chance of detecting aneuploidy given a positive ESS result in a surveillance population and therefore equates to:

$$\frac{Aa}{Aa + Ad + Dd} = 0.27 \text{ or } 27\%$$

### Conclusions:

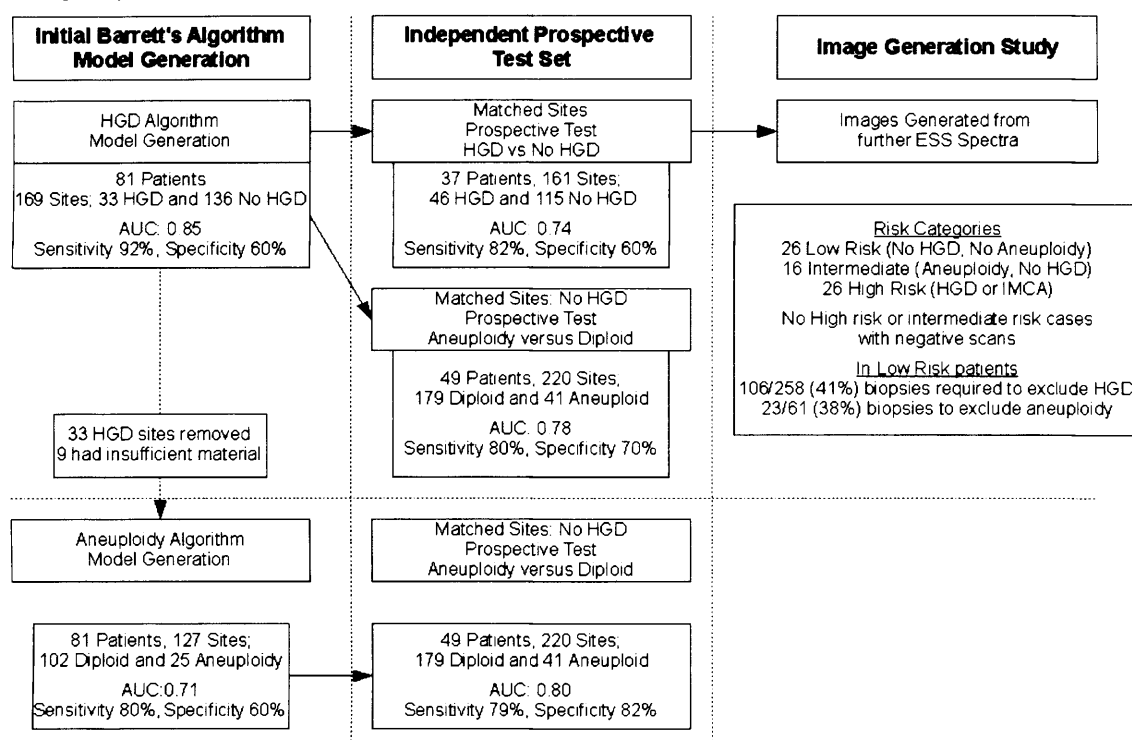
Many of the potential errors associated with the diagnosis of aneuploidy using image cytometry have been discussed in chapter 6 but one further source of error is histogram interpretation. In this study the two observers met to agree the previously described criteria (Section 5.6), independently reported the histograms and then met to agree a consensus in cases of disagreement. A consensus was reached in all cases. Disagreements did occur in 4 cases over the size and position of secondary abnormalities but these were all resolved by consensus and these changes did not alter the overall diagnosis of diploid or aneuploid. This pre-agreement of diagnostic criteria and multiple histogram reporting should have reduced any errors in interpretation.

ESS appears to be able to detect aneuploidy in Barrett's Oesophagus *in vivo* in the absence of HGD with reasonable accuracy. Over 85% of patients undergoing surveillance do not have aneuploidy in their Barrett's oesophagus so if ESS could successfully exclude this abnormality as well as HGD; then these patients would not require a further endoscopy for at least five years. See summary of all ESS studies in figure 7.19 below. Resources could then be focused on the surveillance or even treatment of patients with aneuploidy. This ESS algorithm for the detection of aneuploidy requires prospective testing in a larger dataset in a surveillance population but these results are very encouraging.

In the examples calculated in the results section above with a balanced cut-off chosen between sensitivity and specificity the negative predictive value is again excellent at 99%. The positive predictive value of any positive sites from which biopsies are taken is relatively low at 27% but this would equate to an 82% reduction in the number of biopsies processed from diploid patients

in a surveillance population. In any condition with a low prevalence the PPV falls precipitously unless the specificity is extremely high. These estimated reductions in the number of negative biopsies processed for aneuploidy by targeting with ESS may permit the introduction of screening for aneuploidy into the general population were the ESS results to be confirmed in prospective multicentre testing.

**Figure 7.19:** Summary chart of ESS datasets and the results of testing for the detection of HGD and aneuploidy



The question remains of how best to fit together the three algorithms described above (squamous versus columnar, HGD in Barrett's versus no HGD, and aneuploidy versus no aneuploidy in BO without HGD). As already suggested it is possible to examine the ESS spectra from one site sequentially for BO then aneuploidy and then for HGD. The principal concern with processing ESS spectra through three consecutive algorithms is that false positive errors would be at least additive if not multiplicative. Alternatively a multivariate analysis could be performed. This multivariate analysis would almost certainly be preferable but requires a new level of statistical complexity, is beyond my ability to perform, requires large amounts of computer processing power (up to one week to run a single algorithm generation step) and forms part of Miss Ying Zhu's PhD thesis on advanced statistical analyses of ESS data. This is briefly described in section 7.5.

## **7.4 Additional Technical Problems which arose from the above studies**

### ***7.4.1 Single Pathologist Reporting for HGD***

Pathologists agree a diagnosis of HGD with only a kappa value of 0.8 in the data above. In the HGD algorithm generation study (section 7.3.2) the three pathologists failed to reach a consensus in 4/49 cases (8%) and these were subsequently excluded from the analysis. This was extremely beneficial for algorithm generation in order to ensure that only definite high risk biopsies were in the training set. The criticism of excluding these sites from testing was that these biopsies occur in real clinical practice and ultimately would have to be analysed by the algorithm. These sites where the pathologists disagree about the 'gold standard' are almost impossible to classify without further testing (such as with aneuploidy). The histology in all but the initial HGD algorithm generation study has only been reported by a single, albeit expert, GI pathologist. This does limit the certainty with which the 'gold standard' can be applied but multiple slide reporting on a large scale was simply not possible. This was an accepted weakness of the prospective testing with the possible introduction of a small error but these are the conditions that the ESS system would have to perform under in real clinical practice and most DGH pathologists already operate under.

In the diagnosis of aneuploidy all histograms were agreed by two blinded reporters who had previously agreed criteria for image abnormalities. In cases of discrepancy the reporters resolved these differences by meeting to form a consensus. This hopefully minimised the potential for error in the diagnosis of aneuploidy. Furthermore, the two observers reporting aneuploidy in the ten blinded cases sent from Seattle agreed in all cases with the 'gold standard' reporting from that group.

### ***7.4.2 Co-registration of optical measurements and physical biopsy***

HGD has been shown to be patchy and usually occult within BO (Reid et al. 1988) and on average HGD occupied only 1.3cm<sup>2</sup> out of 32cm<sup>2</sup> of Barrett's (Cameron and Carpenter 1997). Furthermore, only part of a biopsy processed for histology might contain HGD which can be diagnosed if as few as two crypts were abnormal (Novelli MR, 2006). To makes matters worse the interrogation volume of the ESS probe is 1mm<sup>3</sup> much smaller than the size of a biopsy

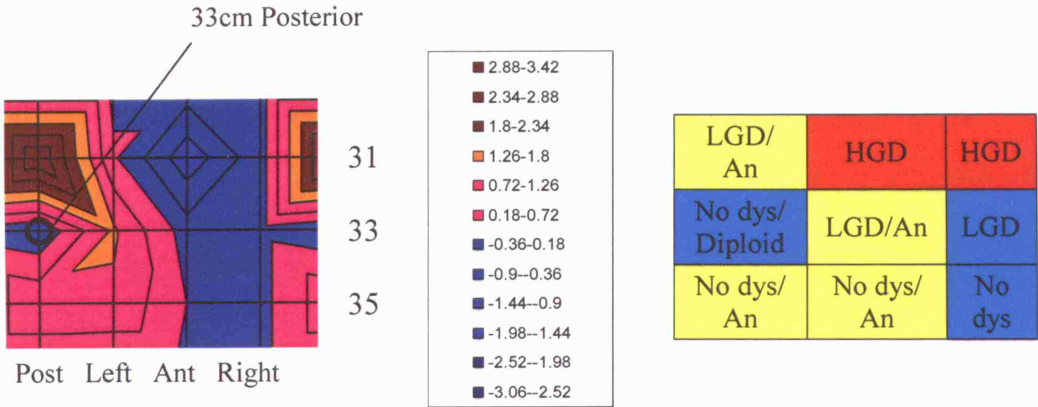
collected using large capacity forceps. Even if the ESS measurement were to be taken from exactly the same spot as the biopsy then useless there was widespread HGD throughout the biopsy then the ESS measurement could have been collected from a part of the biopsy that was non-dysplastic. This is of particular importance in algorithm training as misclassification in this way results in non-dysplastic spectra being used in the HGD training set and for that reason only widespread abnormalities were used for HGD training. This remained a problem, however, for testing but would only result in an underestimation of the true accuracy of ESS. One solution would be to use smaller biopsy forceps more closely approximating the area of optical interrogation but this would run the risk of inadequate material for histological processing and thereby incorrectly fail to diagnose HGD through sampling a smaller area of the BO. As a result of this solution possibly affecting clinical decision making and patient care it was considered unacceptable.

The same problem arose for aneuploidy but to a lesser extent. Aneuploidy has been described as more of a 'field effect' in BO (Maley and Reid 2005; Maley et al. 2004) but was still present only in occult patches. The Seattle group, however, have found situations where aneuploidy was present in only half a biopsy collected (Reid and Rabinovitch 2006) and therefore co-registration errors of this type probably also occurred in the aneuploidy study.

A second aspect to co-registration existed. The exact matching of an optical measurement to a biopsy collected for histology was difficult even for motivated, experienced endoscopists.

**An example of this second co-registration issue:** The site 33cm posterior was negative on the scanned image below (figure 7.20) but a second optical reading collected from 33cm posterior was positive when collected on the same day. The matched biopsy collected for histology was diploid and non-dysplastic but all the surrounding optical measurements were strongly positive and showed either aneuploidy or HGD. It was impossible to know if the optical probe and biopsy were taken from exactly the same position and the result just a false positive or if the position had slipped slightly to a different area between optical measurement and biopsy for histology.

**Figure 7.20: Example of ESS Image demonstrating difficulties with co-registration**



No dys= No dysplasia, LGD= Low grade dysplasia, HGD= High grade dysplasia,  
An= Aneuploidy

Two solutions might have improved this situation. The first was to use a dual-channelled endoscope. With a dual-channel the optical fibre could have been extended from one channel and the biopsy forceps from the other. After optical measurement acquisition a biopsy could have been promptly taken from the same spot under direct vision. This solution would still have been subject to error, albeit less so, particularly due to movement of the oesophagus with respiration or patient movement (coughing or retching). Dual-channelled endoscopes were not available during this project. The more elegant solution offered by our collaborators in Boston but currently still in development is a single optical and biopsy probe that can fit down a single biopsy channel. See figure 7.21. This advance would allow immediate biopsy collection for histology after optical measurement simply by closing the biopsy forceps and would remove the second part of this co-registration error. It might also inadvertently help the initial problem described above because in early testing the biopsy size was also smaller. This does, however, have to be reconciled with not compromising patient care.



**Figure 7.21: Combined Optical Probe and Biopsy Forceps for Histology**



#### ***7.4.3 Fibrous strictures, ulceration and squamous mucosa***

The inadvertent inclusion of squamous sites should, in theory, have been less of a problem because squamous mucosa is visibly different from Barrett's which allowed the endoscopist to target optical measurements away from squamous sites. Curiously histology from 'matched sites' still occasionally demonstrated squamous or mixed squamous and columnar mucosa. It was not clear if this was operator error, lack of accurate co-registration or occult small areas of squamous mucosa which appeared as Barrett's Oesophagus during endoscopy. Irrespective of the cause if the algorithm for HGD was applied to these sites it misclassified 62% of them as high risk. This false positive rate for HGD is potentially very destructive to datasets. Additionally in a surveillance population, where circumferential BO is likely to be less common and Barrett's islands or tongues are more prevalent, a pre-analysis step to automatically exclude squamous spectra would be essential.

The data above (section 7.3.1) with high accuracy (AUC 0.95) for the differentiation between squamous and columnar mucosa have now been incorporated into the new software which allows the real time targeting of biopsies in the endoscopy room. Squamous spectra can now be automatically rejected and measurements recollected if necessary.

Ulcerated sites and fibrous strictures were also not included in these analyses. These visible abnormalities at present continue to require biopsy. The difficulty with ulceration and fibrous strictures was that readings collected from these sites were relatively rare and to include a significant number of these sites for algorithm generation was impossible. This was a weakness

of the above studies with the optical software not trained to deal with these sites and therefore they still required biopsy. Fortunately, targeting biopsies to visible lesions is not difficult.

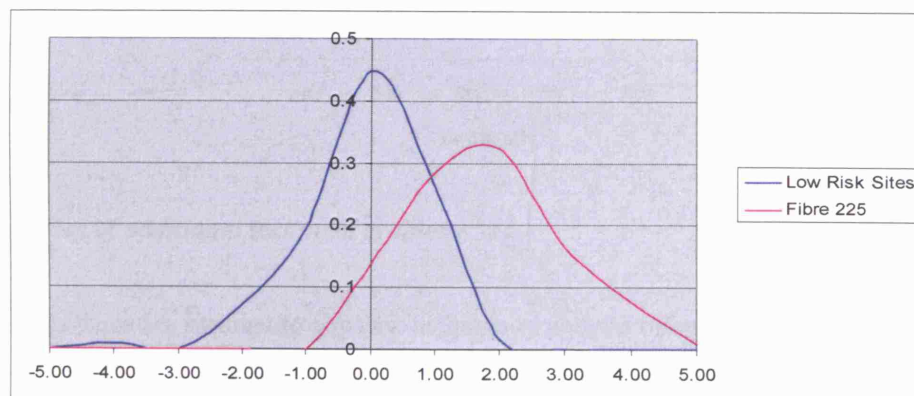
#### 7.4.4 Optical Fibre Quality Control

Continuous monitoring of all the hardware components is of course necessary. From standard monitoring of fibre performance one out of over 30 optical fibres used during this project produced very different optical measurements in a systematic way. This fibre (number 225) consistently measured higher light collection between 450nm-520nm. Figure 7.23. This error was magnified by the standard normalisation of the spectra during analysis and resulted in almost all (85%) of the spectra collected being classified as high risk with a clear shift in the distribution of canonical scores upwards (right shift in graph). Table 7.7 and Figure 7.22. If the spectra were cropped from 525nm to 800nm, excluding this affected region, then the normalised spectra from fibre 225 are then almost identical to all other low risk spectra. Figure 7.24

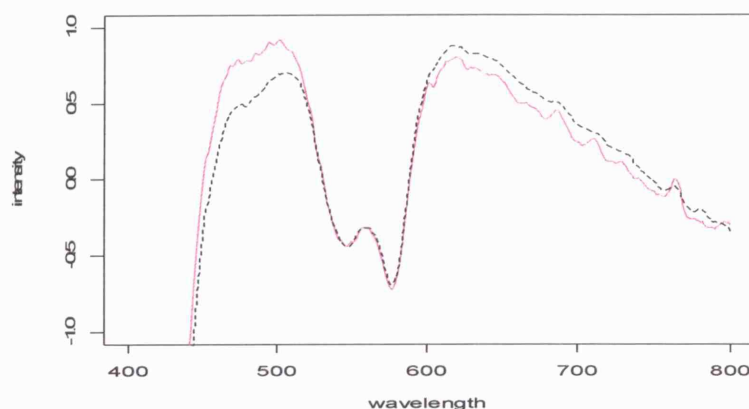
**Table 7.7: Sensitivity and Specificity for Fibre 225 compared to other fibres:**

	<b>Gut Paper <i>Training Set</i></b>	<b>Fibre 225 <i>Prospective Test</i></b>	<b>All fibres <i>Prospective Test</i></b>
Sensitivity	92%	4/4 (100%)	82%
Specificity	60%	9/56 (16%)	60%

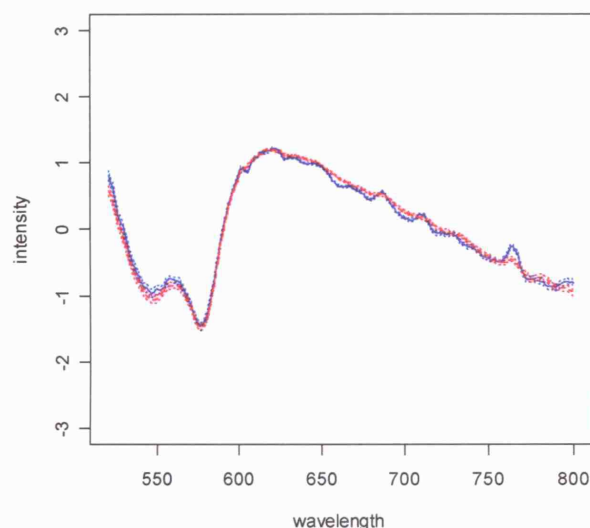
**Figure 7.22: Canonical Scores for low risk sites using Fibre 225 and other fibres in a prospective test:**



**Figure 7.23: Mean spectra from Fibre 225 (Pink) and all other fibres (Blue) using standard pre-processing**



**Figure 7.24: Mean Spectra from Fibre 225 (Pink) compared to all other fibres (Blue) but cropped before normalisation outside 520-800nm**



#### **7.4.5 Summary of additional technical problems**

Technical problems are inherent to any new technology and are often only detected during initial testing. The list above is almost certainly not exhaustive and new problems will be detected in forthcoming studies. None of the above problems have so far proved to be ‘deal-breakers’ over the usefulness of this technology and some appear to have been resolved (eg automatic exclusion of squamous sites). Histology as a gold standard is difficult but this is the current limitation of all clinical practice and while not ideal will remain a problem for all clinicians involved with the

surveillance of Barrett's Oesophagus until a panel of biomarkers becomes routinely available. Fibre quality control, along with ensuring Spectralon cleanliness, requires careful ongoing monitoring with no substitute for good scientific practice. Visible lesions such as ulcerated sites and benign appearing strictures can be targeted for biopsy but with enlarged datasets these might also be interpreted using automated algorithms and therefore analysed prior to biopsy. Finally, and potentially most seriously, co-registration is a complicated problem not only to quantify but also to eliminate. If combined optical probes with biopsy forceps were to become available then these would go a long way to significantly reducing this problem.

### **7.5 Advanced Statistical considerations**

*The novel statistical technique described below was designed and tested by Miss Ying Zhu in the National Medical Laser Centre. The development of this new statistical approach was her work and formed an integral part of her PhD thesis. I am grateful to her for allowing me to summarise her work as it is relevant to the future goals of this project.*

Four replicated ESS measurements were collected from the same site in rapid succession in all the BO studies above. The common variation between the spectra within a site was then removed if these features were consistent across all sites in the dataset irrespective of whether the site was high or low risk. This common variation was shown to be non-discriminatory and equated to the noise of collecting in vivo measurements (for example, angle and pressure of the optical probe) and biological variation not related to disease. After this noise was removed a 'residual' spectra was left. The cleaned residual spectra were then analysed in the normal way by principle component analysis and linear discriminant analysis. This technique was termed 'EROS' or Error Removal by Orthogonal Subtraction.

Ying has shown, in development of this technique, that there was a very slight improvement in accuracy with an AUC of 0.87 for the detection of HGD compared to 0.86 without EROS analysis. This technique only required the use of 7PCs in the LDA. The use of fewer PCs suggested that EROS may potentially be more robust, through less overfitting, in prospective testing.

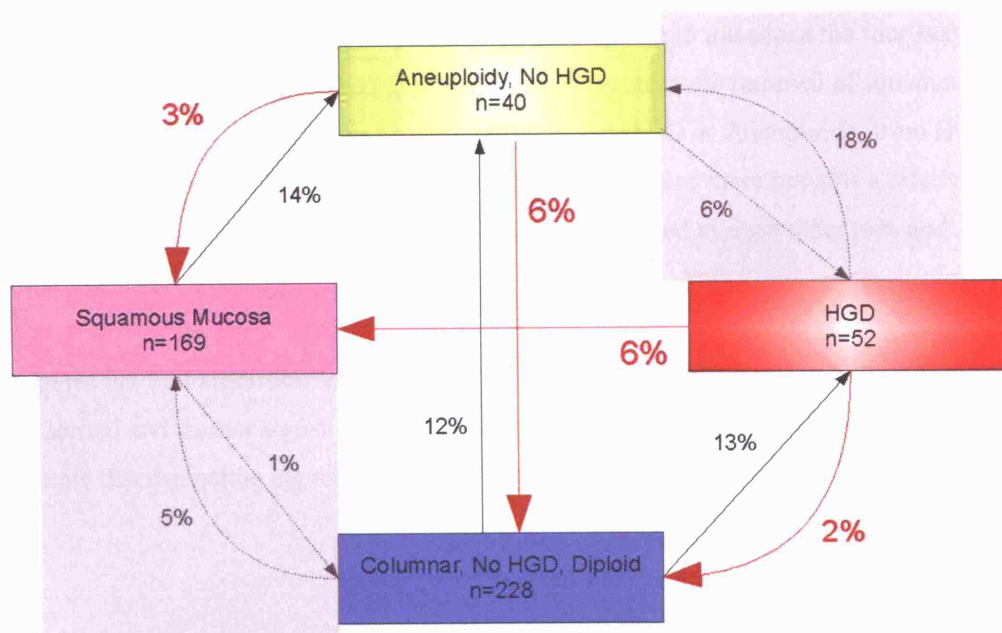
These findings also suggested that further refinement of the statistical techniques may increase the accuracy of this technique to differentiate between low and high risk sites. We have an

ongoing collaboration with Professor Tom Fearn, Head of Statistics UCL to investigate this further.

Ying has also developed a four way multivariate classification. The four groups in this analysis are squamous mucosa (Sq), columnar mucosa without aneuploidy or HGD (low risk), columnar mucosa with aneuploidy (intermediate risk) and columnar mucosa with HGD (high risk). This multiway classification is a far more elegant solution than running three algorithms sequentially. The rather complicated diagram below (figure 7.25) can be simplified using the clinical algorithm of collecting a biopsy in practice if the ESS analysis suggested high or intermediate risk.

1. Sensitivity for HGD sites (biopsy collected if classified as high or intermediate risk) is 91%.
2. Sensitivity for aneuploidy sites (biopsy again collected if classified as high or intermediate risk) is 92%.
3. Specificity of low risk sites being incorrectly diagnosed as intermediate or high risk is 75%.
4. In the rare event of squamous sites being incorrectly analysed there is a 14% false positive rate.

**Figure 7.25:** Four-way multivariate classification for sites collected in vivo in the oesophagus



The arrows demonstrate incorrect classifications and the percentage of sites incorrectly classified in these directions. Biopsies would be targeted in clinical practice to sites with HGD or aneuploidy and therefore the incorrect classifications shaded grey are of less clinical relevance. The arrows and percentages in red represent false negative results

This is just a preliminary analysis and requires further refinement particularly with the addition of EROS and also requires prospective testing but offers a potential solution to multiple outcome variables.

## 7.6 Summary

In essence the above studies have moved our understanding of ESS forward to the point where, with the new software developments now available, the ESS system can provide instant results within the endoscopy room allowing the targeting of biopsies to high risk sites in Barrett's Oesophagus. Not only have we shown that ESS detected HGD, the only widely available biomarker of high risk, but it also detected aneuploidy. These algorithms have been tested separately in independent prospective datasets and the AUC was maintained at 0.76 and 0.80 respectively. Furthermore this chapter demonstrates that an ESS algorithm developed to detect HGD also has a high rate of being positive with sites displaying aneuploidy but not HGD. These data have allowed the addition of an automated pre-processing step for the removal of squamous sites which are 'accidentally' measured with a high degree of accuracy (>95%).

With further advanced statistical processing it may be possible to introduce the four-way multivariate discrimination described above allowing the automatic removal of squamous mucosa and differentiating between Barrett's Oesophagus without HGD or Aneuploidy, from BO with aneuploidy, and BO with HGD. Interestingly in this analysis also there remains a relatively high incorrect classification of aneuploidy and HGD being diagnosed as each other (6% and 18% respectively).

Testing with the first algorithm for the targeting of biopsies *in vivo* in the endoscopy suite has already started and further statistical work is on-going to allow for the addition of a four-way multivariate discrimination algorithm with or without EROS analysis at a future date.

## Chapter 8

# Aminolaevulinic acid Photodynamic therapy to prevent oesophageal cancer and eradicate High Grade Dysplasia in Barrett's Oesophagus

## **8 Aminolaevulinic acid Photodynamic therapy to prevent oesophageal cancer and eradicate High Grade Dysplasia in Barrett's Oesophagus**

*It should be noted that 46 of the patients presented below had received PDT prior to my work starting although some have been retreated by me. Since 1<sup>st</sup> October 2004, all further treatment and all follow up have been performed by me. The collation of results and all statistical analyses have also been performed by me.*

### **8.1 Introduction**

Photodynamic therapy (PDT) is potentially an effective lower risk alternative to oesophagectomy for the treatment of high grade dysplasia (HGD) in Barrett's oesophagus (BO) (Overholt, Panjehpour, and Halberg 2003; Overholt, Panjehpour, and Haydek 1999). In a randomised controlled trial of over 200 patients with HGD in BO, PDT was shown to reduce oesophageal cancer rates by 50% in the PDT group when compared to patients treated with a proton pump inhibitor alone (Overholt et al. 2005).

Photofrin is unfortunately taken up in the oesophagus by both the mucosa and the submucosa and is, therefore, associated with oesophageal stricture formation at a rate of 22% with one treatment and rising to 50% with multiple treatments (Overholt, Panjehpour, and Halberg 2003; Overholt, Panjehpour, and Haydek 1999). These strictures can require up to 6 dilatations. (Panjehpour et al. 2005) In attempts to lower this stricture rate lower light doses have been used to activate the photofrin but as the energy of the laser light is reduced so is the efficacy (Panjehpour et al. 2005). Patients have also been given oral steroids following PDT but this did not reduce stricture formation (Panjehpour et al. 2000). The second principle side effect of Photofrin PDT is light sensitivity for up to 3 months following treatment. There are vastly different rates of serious reactions reported ranging from 1% (Overholt et al. 2005) to 33% of patients; the latter group all required oral steroids for skin phototoxicity (Wolfsen et al. 2004). Up to two thirds of patients treated with Photofrin have reported some form of photosensitivity reaction (Overholt, Panjehpour, and Haydek 1999). These complications significantly affect the acceptability of Photofrin PDT.

5-aminolaevulinic acid (ALA) is potentially a lower risk alternative. Skin photosensitivity only lasts 1 day with PPIX plasma concentrations undetectable after 10 to 12 hours. Additionally,



because far more protoporphyrin IX is produced in the gastrointestinal mucosa than in the underlying muscle, the risk of stricture formation is minimal (Regula et al. 1995;Loh et al. 1996). The PDT effect, however, is limited to a depth of 1-2mm, so it is ineffective for invasive cancer (Barr et al. 1996;Gossner et al. 1998). These advantages over Photofrin may be important to patients as ALA-PDT appears to carry no long term risks.

There have, however, been many suggested regimens of ALA-PDT for the treatment of dysplasia arising in BO with highly variable results (Pech et al. 2005;Gossner et al. 1998;Ackroyd et al. 2003;Ackroyd et al. 2000a;Kelty et al. 2004b;Kelty et al. 2004a;Hage et al. 2004;Peters et al. 2005a;Barr et al. 1996). The most encouraging case series of 35 patients with HGD in BO reported an 89% (31/35) eradication rate without recurrence at median follow up of 3 years (Pech et al. 2005). This must be compared to the disappointing results of a different study reporting only a 55% (11/20) long term eradication rate with PDT combined with EMR for HGD in Barrett's (Peters et al. 2005a). The PDT regimens used to treat all grades of dysplasia in these studies varied in the amount of energy used to activate the photosensitiser ('light dose'), the amount of oral ALA given prior to PDT (30-60mg/kg) and the wavelength of light used to activate the photosensitiser (red or green light).

Firstly, considering light dose used, there has been activation from as little as 60 J/cm<sup>2</sup> (Ackroyd et al. 2003;Ackroyd et al. 2000a) through 100 J/cm<sup>2</sup> (Peters et al. 2005a;Hage et al. 2004) and up to 150 J/cm<sup>2</sup> (Forcione et al. 2004;Barr et al. 1996;Gossner et al. 1998;Pech et al. 2005). Depending on the size of the balloon used to deliver the light into the mucosa of the oesophagus the maximum light delivery to date has been around 1000J/cm of Barrett's length (150J/cm<sup>2</sup> through a 20mm device) (Gossner et al. 1998). It is hardly surprising that these studies have reported very different success rates for PDT. Furthermore, none of these studies have compared the relative efficacy of different doses of light in the same cohort of patients holding the other parameters constant. Finally, in two studies treating low-grade dysplasia good eradication rates were achieved up to 95-100% using green light at much lower light (264J/cm) and drug (30mg/kg) doses (Ackroyd et al. 2003;Ackroyd et al. 2000a). This raises interesting questions as to whether such high doses of ALA are really required for the treatment of HGD or if green light, more avidly absorbed by the mucosa than red light, is a more effective activator of PPIX.

Studies are necessary to compare the effect of different parameters of ALA-PDT prior to large randomised controlled trials comparing the efficacy of ALA to other photosensitisers such as porfimer sodium or other modalities such as argon plasma coagulation (Hage et al. 2004;Kelty et al. 2004b). Of interest, these studies have only been performed for porfimer sodium recently in an attempt to reduce oesophageal stricture formation (Panjehpour et al. 2005).

Studies aimed at assessing the importance of light dosimetry, dose of ALA and wavelength of light used to activate the photosensitiser are warranted in order to determine the efficacy of ALA-PDT for HGD arising in BO.

**Table 8.1: Studies to date with ALA-PDT for Barrett's Oesophagus**

Author, Year	n	Hist	Aim	ALA Dose mg/kg	Light Dose J/cm	Red or Green	Outcome	Comments
Hage, 2004	26	No dysplasia	RCT PDT vs APC BO Reversal	60	785 +/- Fractionated	Red	PDT 82% Fractionated 90% APC 67%	Follow up 12m 1 Death
Kelty, 2004	34	No dysplasia	RCT vs APC BO Reversal	30	668	Red	Equal 50% Ablation	24% Buried Glands
Kelty, 2004	25	No dysplasia	RCT 30 v 60 BO Reversal	30 v 60	668	Red	No difference	PPIX level NS different
Ackroyd, 2003	40	LGD	Cohort, LGD Eradication	30	264	Green	38/40. (95%) Eradication LGD	53 months follow up
Ackroyd, 2000	36	LGD	RCT PDT vs PPI	30 v PPI	264	Green	Success: PDT 18/18(100%) PPI 6/18 (33%)	
Barr, 1996	6	HGD	Vary Light Dose	60	Max 660	Red	6/6 Success	2/6 Buried Glands
Peters, 2005	33	HGD/ IMCA	EMR then 20 PDT	40	785	Red	11/20 (55%) PDT Success	
Forcione 2004	6	HGD/ IMCA	Eradicate lesion	30	150 J/cm <sup>2</sup> *	Red	(1/5) 20% Success	1 Death
Gossner, 1998	28	HGD/ IMCA	Eradicate lesion	60	948	Red	Success HGD 9/9 IMCA 10/19	17 months follow up
Pech, 2005	66	HGD/ IMCA	Eradicate lesion	60	150 J/cm <sup>2</sup> *	Red	Success HGD 31/35 (89%) IMCA 21/31 (68%)	60 months Follow up

\* No Balloon Size Reported to calculate energy into the tissues

## **8.2 The effect of light dose in ALA-PDT on the eradication of High Grade Dysplasia in Barrett's Oesophagus**

*This study has been published as "How light dosimetry influences the efficacy of photodynamic therapy with 5-aminolaevulinic acid for ablation of high-grade dysplasia in Barrett's esophagus."*

*Mackenzie GD, Jamieson NF, Novelli MR, Mosse CA, Clark BR, Thorpe SM, Bown SG, Lovat LB. Lasers Med.Sci. 23.2 (2007a): 203-10.*

### **8.21. Aims**

This study aimed to assess the importance of light dosimetry in determining the efficacy and safety of ALA-PDT for HGD arising in BO.

#### **8.2.2 Methods**

This non-randomised light dose escalation study was approved by the joint UCL/UCLH committee on the ethics of human research and all treated patients gave written, informed consent.

##### *Patient Assessment*

Subjects were referred to the National Medical Laser Centre at the University College London Hospitals for the management of Barrett's associated HGD between 1998 and 2002. All patients were assessed initially by one operator (LBL) at day case endoscopy. The extent of BO was formally mapped, together with evidence of nodules, by recording the presence of columnar, squamous or mixed mucosa in each quadrant for every centimetre of BO. Four-quadrant large-cup biopsies were taken at levels every 2 cm throughout the columnar-lined oesophagus and targeted from suspicious lesions. Endoscopic ultrasound (EUS) was used to examine the Barrett's segment to assess any visible abnormalities and look for lymphadenopathy. If there was suspicion of lymph-node metastasis, helical CT scanning of chest and abdomen and 5-FDG positron emission tomography (PET) scans were performed and the results reviewed at a Multi-Disciplinary Meeting. All patients received proton pump inhibitors.

The slides from the referring hospital showing HGD were reviewed at UCLH by a consultant histopathologist (MRN) with a specialist interest in Barrett's oesophagus, who also examined all biopsies from the initial and follow up endoscopies at UCLH. The Vienna classification was used

to categorise dysplasia (Schlemper et al. 2000). In all cases two independent pathologists confirmed the presence of HGD (Vienna 4.1-4.3). Patients were counselled about benefits and risks of surgical treatment. Only patients declining surgery, or considered too high a surgical risk because of co-morbid conditions were included in the study. Patient exclusions are listed below.

**Table 8.2:** Exclusion Criteria for entry into the light dose study.

Histological evidence of invasive cancer
Radiological evidence suggestive of tumour stage T1N0 or higher by endoscopic ultrasound, computerised tomography (CT) or positron emission tomography (PET) scan assessment, even in the absence of histological proof of invasive cancer
Symptomatic ischaemic heart disease (exertional angina) at the time of entry to the study
History of chronic liver disease
Abnormal liver biochemistry present prior to entry - (ALT, ALP or bilirubin greater than twice the upper limit of normal)
Less than 18 years old
Pregnancy
Diagnosed with porphyria

#### *Photodynamic therapy*

ALA (99% purity) was supplied by DUSA Pharmaceuticals, New York, USA. All patients were admitted to hospital and counselled to avoid exposure to bright artificial or natural light for 36 hours after administration of ALA. Intravenous fluid infusion was started 8-12 hours prior to treatment in all but the first 5 treatments and an antiemetic (granisetron 1mg) given intravenously 15 minutes before ALA. ALA was administered orally in three doses of 20mg/kg diluted in 50 ml water at 5, 4 and 3 hours prior to light delivery to reduce the risk of patients vomiting the photosensitiser (Regula et al. 1995).

#### *Light delivery*

Endoscopy was performed under conscious sedation with midazolam (2-10mg) and pethidine (25-50mg). We initially used 25mm Wizard X-Cell balloons for light delivery (Wilson-Cook, USA) inflated with 35 ml water, but later replaced these by flexible silicone bolsters (diameter 16mm) designed and manufactured in our department of medical physics (Lovat et al. 2005). The delivery device was passed over a guide-wire into the oesophagus to the required distance. The guide wire was removed and a cylindrical diffuser fibre (Biolitec, Germany) matching the length of the delivery device was passed through the guide-wire channel so that it lay centered within the device at the level of the treatment area. Light was delivered from a semi-conductor laser (Diomed Ltd, Cambridge UK). All treatments in this study were performed using a laser emitting

at 635nm. Up to 7cm of Barrett's could be treated with a single fibre; if the Barrett's segment was longer then treatments overlapping by 0.5cm were performed.

#### *Light dose*

The light dose can be expressed as either J/cm length or J/cm<sup>2</sup> of the surface area of the balloon. With doses expressed as J/cm<sup>2</sup> total light dose varies if the size of the balloon is different which makes comparing studies difficult. Light doses in this study are, therefore, expressed in J/cm of oesophagus treated. Light was administered as either 500J/cm of treated oesophagus (low dose, equivalent to 100J/cm<sup>2</sup> using a 16mm balloon), 750J/cm (medium dose, 150J/cm<sup>2</sup>), 1000J/cm (high dose, 200J/cm<sup>2</sup>) or two 1000J/cm treatments given one month apart (highest light dose, 400J/cm<sup>2</sup>). The fluence rate of laser power to the surface was 100mW/cm<sup>2</sup>. The 1000J/cm treatments were well tolerated but because of the long duration of treatment (33.3 minutes per treated segment at 1000J/cm) it was felt inappropriate to give a light dose of more than 1000J/cm per segment at any one time. In the highest light dose group, 1000J/cm of light was given at each of two sessions scheduled a month apart.

#### *Follow-up*

The treatment effect was assessed at endoscopy 24-48 hours after PDT. Once eating and drinking satisfactorily, the patient was discharged (usually 48 hours after PDT) with oral analgesics for any residual odynophagia. Patients were followed with endoscopic quadrantic large-cup biopsies every 2cm throughout the length of the original Barrett's segment at 1, 3, 6, 9 and 12 months after PDT, 6 monthly for the next year then at yearly intervals thereafter. The length and extent of the Barrett's segment was again formally mapped 3-6months after PDT to assess squamous re-epithelialisation and compared to biopsies to ensure accuracy. If HGD was found during follow-up, further therapy (PDT or other) was considered at a multi-disciplinary team meeting.

#### *Outcome Measures*

Treatment success was defined as complete and continuing eradication of high grade dysplasia in quadrantic biopsies taken every 2cm of treated oesophagus at all time points during follow-up. Treatment failure was defined as detection of high grade dysplasia or cancer at any time following PDT.

#### **Statistical Methods**

Kaplan Meier analysis and log rank score were used to assess the correlation between light dose and treatment response.

### 8.2.3 Results

Between August 1998 and June 2002, 24 patients with high grade dysplasia (19 men, 5 women) were recruited to this study. Median age at treatment was 73 years (range 52-87 yrs). There was no significant difference between the groups with regard to either age, length of Barrett's or extent of HGD (Table 8.3). Those patients that failed initial therapy were offered further treatment with either other therapy or further PDT. Median follow up is 45 months (1-78 months).

**Table 8.3:** Pretreatment data for light dose groups

	n	Median Age† (Range)	Median Barrett's † Length (Range)	Focal HGD†	Diffuse HGD†
Highest Light Dose (1000J/cm x2)	8	72 (52-87)	5cm (1-10)	4	4
High Light Dose (1000J/cm)	2	69 (64-74)	4cm	2	0
Medium Light Dose (750J/cm)	9	73 (59-86)	5cm (3-12)	5	4
Low Light Dose (500J/cm)	5	75 (63-77)	6cm (5-10)	2	3

Focal HGD defined as HGD present at single biopsy level.

† No significant differences within these groups, p=NS

#### *Effect of light dose*

The highest light dose was significantly better than low and medium light dose for the eradication of HGD in Barrett's oesophagus (Log rank test,  $p < 0.01$ ). There is a clear correlation between the light dose delivered and the treatment response (Table 8.4 and Figure 8.1). Six out of eight patients (75%) treated with the highest light dose (1000J/cm x2) compared to 1/2 (50%) with a single high light dose treatment, 2/9 (22%) receiving medium light dose and 0/5 (0%) receiving low light dose had successful long-term eradication of HGD.

**Table 8.4:** Treatment results of ALA-PDT dependent on light dose used

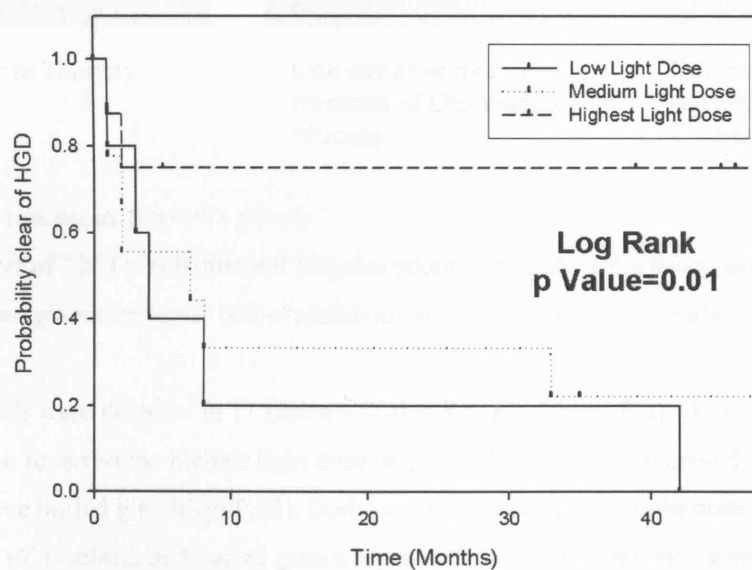
	n	Mean Barrett's Regression	Patients with Buried Glands	Biopsies with Buried Glands	Developed Cancer	Clear of HGD & Cancer during Follow up
Highest Light Dose (1000J/cm x2)	8	40% *	2 *	3/512 (0.6%)*	1	6 ♦
High Light Dose (1000J/cm)	2	50%	0	0/80 (0%)	0	1
Medium Light Dose (750J/cm)	9	10%	7	22/340 (6.5%)	3	2
Low Light Dose (500J/cm)	5	15%	2	3/332 (1%)	2	0

\* Significantly different from low/medium light dose groups,  $p < 0.05$ .

♦ Significant difference compared to low/medium light dose groups, log rank test,  $p = 0.01$

**Figure 8.1:** Time to relapse of high grade dysplasia after PDT at different light doses

The highest light dose was associated with a much better response than either medium or low light dose.

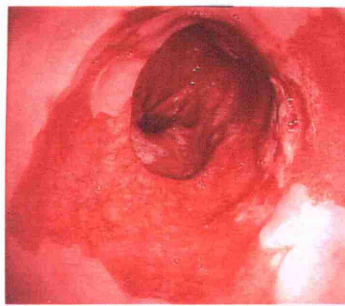


(The two patients treated with a single high light dose treatment are not represented on the Kaplan Meier because of small numbers.)

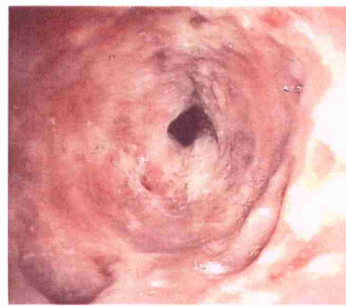
### *Reduction in Barrett's length*

Reduction in length of the Barrett's segment was a secondary endpoint of this study. Pre-treatment, the median length of Barrett's mucosa was 6cm (range 1-12cm). After treatment, reduction was highly variable ranging from 0-100% but significantly greater in the highest light dose group compared to low/medium light doses. The mean reduction in Barrett's was approximately 40% and 15% respectively ( $p<0.05$ ). Two patients had complete ablation of their Barrett's segment. Both had received the highest light dose. Figure 8.2 shows a patient's Barrett's pretreatment, one day after PDT and following healing at one month.

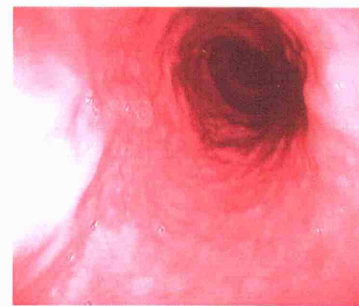
**Figure 8.2: Oesophageal Mucosa before and after ALA-PDT**



Prior to Therapy



One day after treatment:  
Necrosis of Oesophageal  
Mucosa



One month later: Squamous  
re-epithelialisation  
Dysplasia eradicated

### *Buried sub-squamous Barrett's glands*

Out of a total of 1264 pinch mucosal biopsies taken during the follow-up period, 28 (2.2%) demonstrated glandular tissue below squamous surface epithelium - 'buried glands'.

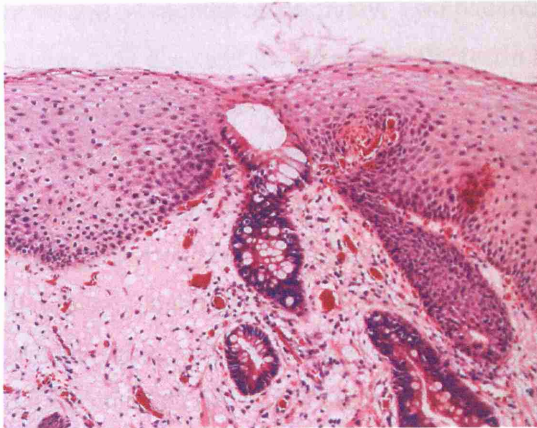
Buried glands were detected in 11 patients (Table 8.4 and Figure 8.3). Only two out of the eight patients who received the highest light dose compared to 9/14 who received 500-750J/cm were found to have buried glands ( $p<0.05$ ). Both patients in the highest light dose group had successful eradication of dysplasia and buried glands were non-dysplastic and only seen at the first (1 month) endoscopy. Neither patient in the 1000J/cm group had buried glands detected. Prolonged follow-up showed no other buried glands (dysplastic or non-dysplastic) at any other time in these groups. Dysplastic buried glands were found in three cases, all of whom had received the medium light dose. This high rate of buried glands in the medium/low light dose groups (64% of patients)



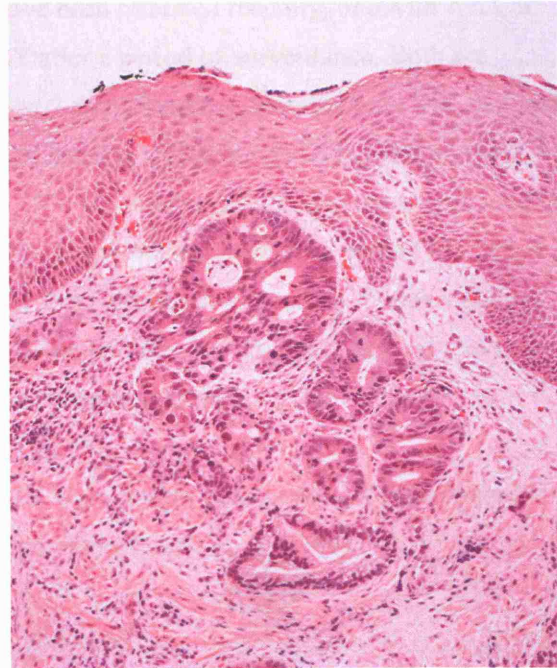
compared to 25% of patients in the highest light dose group is further evidence of the potential inadequacy of lower light doses.

**Figure 8.3: Glands buried under squamous re-epithelialisation post PDT**

3a) Non dysplastic buried glands



3b) Buried Glands with high grade dysplasia



#### *Treatment Failure*

A total of 15 patients failed therapy. All 3 treatment failures in the highest and high light dose group and 60% (7/12) of failures in the other light dose groups occurred early following treatment and were detected at one of the two endoscopies performed within 3 months of PDT. Relapse, more than 3 months after therapy, occurred later in 5 patients treated with low or medium light dose (at 7, 8, 8, 33 and 42 months).

#### *Outcome of patients failing treatment*

Two patients failed ALA-PDT in the highest light dose group. One was found to have an early stage cancer (T1N0) at first follow up which was likely to have been present, but occult, at the time of PDT. This was subsequently successfully treated with chemoradiotherapy and he is well at 48 months. The other had recurrent HGD in a visible nodule treated by an endoscopic mucosal resection followed by successful PDT using meso-tetrahydroxyphenyl chlorine (mTHPC or

Foscan) for residual HGD and is well at 44 months follow up. One patient in the high light dose group failed ALA-PDT and remains under surveillance.

Twelve out of 14 patients in the low or medium light dose group had persistent or recurrent HGD or were found to have an invasive cancer after ALA-PDT. Two patients with cancer (1 and 8 months post therapy) have been re-treated with PDT using mTHPC and are currently in remission at 48 and 52 months respectively. Two patients have been retreated recently, one with ALA at higher light dose and the other with Photofrin PDT after a period of surveillance. Both are currently in remission although follow up is short at 7 and 14 months. Two patients with residual HGD and two with cancer (at 2, 6 months and 8, 14 months respectively) underwent an Ivor-Lewis oesophagectomy and are now in remission. The other two patients who developed locally invasive cancer have been treated successfully with chemoradiotherapy. One patient has had an endoscopic mucosal resection of the tumour and is also currently in remission. The last patient remains under surveillance for HGD, with a view to more radical treatment if clinically indicated. All patients that developed oesophageal cancer were staged as T1N0.

Additionally, the rates of cancer development in the highest light dose group (1/8) compared to lower light doses (6/14) is significantly lower in long-term follow up ( $p < 0.01$ ).

### *Complications*

There were no skin photosensitivity reactions and no oesophageal strictures have developed.

Two patients treated early in our series developed clinically significant hypotension before light delivery. Both patients had been starved for at least 8 hours prior to ALA administration. In one patient, uncomplicated syncope led to PDT being re-scheduled. In another patient hypotension precipitated myocardial ischaemia due to unsuspected coronary artery disease. Several months after successful coronary artery stenting he received PDT using mTHPC and is not included in our analysis of efficacy. We subsequently arranged for all patients to be hydrated intravenously for at least 8 hours prior to ALA administration and have had no further problems with hypotension.

Nausea and vomiting were frequent following PDT, occurring in nearly two-thirds of patients despite antiemetic pre-medication with granisetron. This typically occurred 2-3 hours after PDT was completed and is likely to be a reaction to ALA-PDT. Vomiting lasted less than 24 hours in

all cases. Chest discomfort was common for several days after treatment but was usually well controlled with simple analgesia. Alanine transaminase (ALT) became elevated in most patients after PDT, but in all cases was asymptomatic and returned to normal within a week of drug administration. One patient developed aspiration pneumonia. This was probably related to a very prolonged treatment in a recumbent position in a 12cm segment of Barrett's oesophagus.

#### **8.2.4 Discussion**

Oesophagectomy for an elderly patient with Barrett's-associated HGD has significant associated morbidity and mortality but it seems sensible to aim for cure whilst disease remains confined to the mucosa. This awkward problem has spurred the development of techniques for non-surgical mucosal ablation.

PDT is a promising option as it is possible to treat extended areas of mucosa uniformly. The risk of perforation is extremely low as the submucosal collagen scaffold remains unaffected (Barr et al. 1987). Treatment can be repeated if necessary and other therapeutic options remain open. PDT with porfimer sodium (Photofrin) is an approved treatment although it has the significant problems of skin photosensitivity that may last for weeks and a considerable risk of oesophageal strictures, which may require multiple dilatations (Overholt, Panjehpour and Halberg 2003). PDT with ALA avoids both these problems. We achieved 75% clearance at a median follow up of 40 months in patients treated with the highest light dose, in contrast to the poor results with low and medium light doses.

One of the most difficult problems with PDT is correct light dosimetry. Light dose has been poorly addressed in clinical reports with values that appear to be arbitrary or extrapolated from animal studies. PDT with porfimer sodium is a licensed treatment, yet light dose ranging studies have not been carried out in humans until recently (Panjehpour et al. 2005).

The diameter of the centring balloon used for light delivery in the oesophagus differs between reports. The treated surface area changes with the size of the centring balloon although the total number of cells treated is unchanged. This creates difficulties when defining the light dose as J/cm<sup>2</sup> and in comparing different studies. We suggest, therefore, that it is more appropriate to describe the light dose per centimetre length of treated oesophagus.

Nevertheless, there are other considerations related to the distribution of light within the lumen of the oesophagus and within the mucosa. The light fluence rate in the lumen is often higher than the rate delivered from the fibre due to multiple reflections from the oesophageal wall. Of course, this cannot increase the total light energy available but it can change the distribution of light within the wall of the oesophagus. If reflection is high, the average angle at which light enters the mucosa is close to tangential, so the path length of light within the mucosa is longer than if reflection is low, when the incident angle is closer to perpendicular to the mucosal surface. Bearing in mind that the penetration depth of the light used for ALA-PDT (635nm) is 2-3 mm and the mucosal thickness is only of the order of 0.5mm, the effect of internal reflections in the lumen of the oesophagus can be quite dramatic. Considerably more light will be absorbed in the mucosa when there is more reflection within the oesophageal lumen. In the future, it may be necessary to monitor the light fluence in the lumen to allow for this effect in deciding on the appropriate total light dose to deliver (Van Veen et al. 2002;Stringer MR et al. 2006).

In addition, distending the oesophagus will make the mucosa thinner, which will also change the percentage of the delivered light that is absorbed in the mucosa rather than in deeper layers of the oesophageal wall. Yet another consideration is the fluence rate for light actually absorbed in the tissue. It is well documented for many photosensitisers (including ALA) that the PDT effect is greater (more tissue destruction per joule of absorbed energy) if the fluence rate is lower (Tsutsui et al. 2002;Curnow et al. 1999). Thus it is clear that dosimetry for oesophageal PDT is complex, much of which is beyond the scope of this thesis. It would control one variable if all treatments were undertaken with a fixed diameter of light delivery device, but in the absence of this, we consider defining the light dose in J/cm is more appropriate than  $\text{J}/\text{cm}^2$ .

From our current results, we would not recommend low or medium light doses for treating HGD with ALA-PDT. Our view regarding one light dose of 1000J/cm or two doses given one month apart is that after a single treatment at 1000J/cm it is appropriate to re-biopsy the patient prior to a second ALA-PDT to determine if repeat treatment is necessary.

The safety of any new technique is of crucial importance. No patient in our series has developed an oesophageal stricture or phototoxicity and none have developed advanced oesophageal cancer (all staged T1N0). There was one aspiration pneumonia and we are now careful to treat all patients semi-recumbent rather than completely flat. After our initial experience of profound hypotension in 2 patients, all subsequent patients were well hydrated prior to ALA administration

and remained in hospital for at least 24 hours after treatment. We ensured all patients were able to eat and drink prior to discharge and no further problems occurred. Two other groups who recently presented their initial experience with ALA-PDT reported one fatality each (Forcione et al. 2004; Haringsma, Siersema, and Kuipers 2004). Both patients were treated as outpatient day cases. The cause of death was aspiration pneumonitis in one (Forcione et al. 2004) and not determined in the other, but was within a day of treatment. This death may also have been related to inadequately treated hypotension or aspiration pneumonia.

Buried Barrett's epithelial glands underneath regenerated squamous epithelium have been reported with all ablative therapies used in Barrett's oesophagus and occasionally, such glands can become dysplastic or even malignant (Overholt, Panjehpour, and Halberg 2003; Overholt et al. 2005; Van Laethem et al. 2000; Van Hillegersberg et al. 2003). In our series, a higher incidence of buried glands was associated with the use of lower light doses. Two out of the ten patients who received the highest or high light dose compared to 9/14 who received lower light doses were found to have buried glands ( $p < 0.05$ ). In the two patients with buried glands treated with the highest light dose, these were only found very early after PDT when healing might not yet have been complete. The overall rate of buried glands in this study was 46% of patients, which while higher than in some reports, is comparable to many other studies of ablative therapies including one study of Photofrin PDT (Basu et al. 2002; Ban et al. 2004; Ackroyd et al. 2004). The percentage of the total number of follow up biopsies showing such glands was only 2.2%; it is likely that the more biopsies taken during follow up, the more buried glands that will be found. Occasionally with PDT complete replacement of Barrett's with squamous mucosa occurs within the crypts. This is by far the most desirable outcome but at present cannot be achieved homogeneously across the Barrett's segment and without a significant treatment innovation to provide this uniformity of effect buried glands are likely to remain a problem with PDT.

Perhaps more worryingly, dysplastic buried glands were found in three cases in this series all of whom had received the medium light dose. One of these patients later developed a subsquamous adenocarcinoma. This is, therefore, an important potential cause of treatment failure that is difficult to detect, as it requires multiple biopsies of areas of regenerated squamous epithelium. We have also detected buried glands in patients who had not had any ablative therapy but had been taking proton pump inhibitors (unpublished data) and this has been the experience of other groups (Hornick et al. 2005). A reliable way of detecting buried glands that is simpler than multiple random biopsies is required.

In conclusion, ALA-PDT is a promising approach to treating HGD in Barrett's oesophagus that avoids the main problems associated with porfimer sodium PDT. A high light dose is required (at least 1000J/cm) and it is essential to treat the full length of the Barrett's segment adequately. Careful follow-up is needed to ensure that failures and relapses are detected early. Further studies are now required to compare other treatment parameters used for ALA-PDT in the treatment of high grade dysplasia in Barrett's Oesophagus.

### **8.3 Randomised Controlled Trial of Red versus Green Light Activation of Aminolaevulinic acid Photodynamic Therapy for High Grade Dysplasia in Barrett's Oesophagus.**

#### **8.3.1 Introduction and Aims**

At the time of study design in 2003, there had been only a few studies examining ALA-PDT and the parameters of its use. In a cohort study of 10 patients with HGD all ten were successfully treated using 60mg/kg of ALA activated by red laser light at a dose approaching 1000J/cm diffuser fibre but follow up was short at 10 months (Gossner et al. 1998). In two studies treating low-grade dysplasia excellent eradication rates were achieved at up to 95-100% using green light with much lower light (264J/cm diffuser fibre) and drug (30mg/kg) doses (Ackroyd et al. 2003; Ackroyd et al. 2000b). Data from our own unit at the time suggested that the overall success rate with ALA-PDT activated by red laser light was 57% over a 2 year follow up somewhat worse than the previous data published by Gossner et al (Mackenzie et al. 2007). These studies therefore raised the following questions as to whether green light, more avidly absorbed by the mucosa with a consequent shorter depth of penetration, was a more effective activator of PPIX than red laser light and if lower doses of ALA could be used for the treatment of HGD.

The study above demonstrated that lower light doses (<1000J/cm) are less effective for the eradication of HGD in BO. This RCT aimed to assess the difference in eradication of HGD rates and subsequent oesophageal adenocarcinoma development between different wavelengths of activating light and if a dose of 30mg/kg of ALA is effective.

#### **8.3.2 Methods**

This RCT was approved by the joint UCL/UCLH committee on the ethics of human research and conformed to good clinical practice. In this randomised controlled trial the sample size calculation was based on an expected difference of 35% in the success rates between the study groups (95% with green light and 60% with red light). At a power of 80% and statistical significance of 0.05, the required sample size of 34 patients was calculated.

#### **Patient Selection**

Patients referred to the National Medical Laser Centre at University College Hospital for management of HGD in BO were screened for enrolment between 2003 and 2005. The patients

were assessed initially at day case endoscopy and quadrantic biopsies taken every 2cm throughout the Barrett's segment with additional biopsies taken from any nodules or ulcers. The extent of BO was formally mapped, together with evidence of nodules, by recording the presence of columnar, squamous or mixed mucosa in each quadrantic for every centimetre of BO. Endoscopic ultrasound (EUS) was used to assess any suspicious areas. If there was a possibility of invasive disease the patient underwent CT scanning and 5-FDG positron emission tomography (PET) to exclude invasive adenocarcinoma prior to therapy. All patients received proton pump inhibitors.

The Vienna classification was used to categorise dysplasia (Schlemper et al. 2000) and HGD was confirmed by at least two independent histopathologists. Any discrete nodules of HGD were removed by endoscopic mucosal resection and only patients where residual HGD was confirmed on repeat biopsy in the absence of a visible nodule were eligible to be enrolled. The modified exclusion criteria from the study above are listed in table 8.5 below.

**Table 8.5: Modified Exclusion Criteria for RCT of Red versus Green Light**

Presence of invasive carcinoma of the oesophagus.
History of severe cardiovascular disease, congestive heart failure, or recent syncope of cardiovascular origin.
Patients presenting with abnormal cardiac signs or symptoms and signs of congestive heart failure on physical examination.
Patient less than 18 years old
Pregnancy
Patients who have a history of porphyria, or hypersensitivity to porphyrins.
Patients with a WBC $<2 \times 10^9/L$ .
Patients with a platelet count $<50 \times 10^9/L$ .
Patients with a prothrombin time $>1.5$ times the upper limit of normal.
Patients with impaired renal (creatinine $>200 \mu\text{mol/L}$ ) or hepatic function at time of entry into the study (ALT, ALP or bilirubin greater than twice the upper limit of normal)
Patients were not allowed to receive concurrent chemotherapy, or radiation therapy or chemotherapy within 4 weeks of entry into this study

### **PDT Parameters**

**Dose of ALA:** ALA was supplied by DUSA Pharmaceuticals, New York, USA. Patients were admitted to hospital and given intravenous fluids 8 hours prior to photosensitisation. An antiemetic (intravenous granisetron 2mg) was given 30 minutes before the ALA was taken orally. ALA was initially administered as a single dose at 30mg/kg diluted in water 4 hours before treatment. Following interim data analysis after 16 patients had been recruited (see below) and discussion with the ethics committee the study was stopped and restarted using 60mg/kg of ALA.



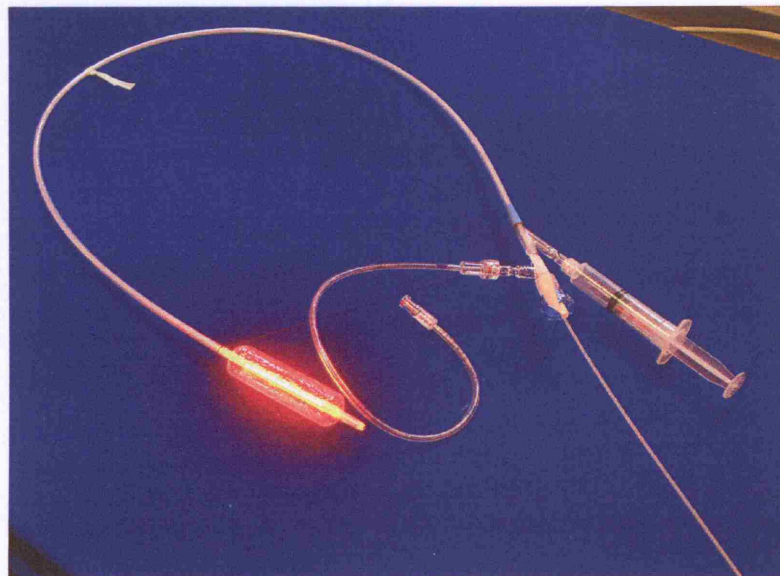
60mg/kg was administered in three divided doses of 20mg/kg diluted in 50 ml water in order to minimise side effects (particularly vomiting) and ingested 5, 4 and 3 hours before light activation.

**Wavelength of Activating Light:** Green light (512nm) was delivered by a Copper Vapour Laser (Oxford Lasers, Oxford, UK) and red light (635nm) delivered by combining into a single optical fibre the light from three Diomed Diode Lasers (Cambridge, UK). Patients were stratified for the length of Barrett's oesophagus requiring treatment ( $>5\text{cm}$  or  $\leq 5\text{cm}$ ) and were then randomised to red or green laser light.

### Photodynamic therapy

Patients were sedated for endoscopy with midazolam 2-10mg and fentanyl 50-150 $\mu\text{g}$ . The proximal and distal margins of the Barrett's segment were noted and used to calculate the desired balloon and diffuser positions. A wire was placed into the stomach at endoscopy and a balloon inserted into the oesophagus to the marked distance (Figure 8.4). Up to 7cm of Barrett's could be treated by a single diffuser fibre, but in the case of longer segments then the treatment could be repeated immediately up to a total of 13.5cm in one endoscopy session (allowing for a 0.5cm overlap). The light dose used was 1132J/cm (or 200J/cm<sup>2</sup> in an 18mm diameter balloon) at a fluence rate of 565mW/cm of diffuser fibre (100mW/cm<sup>2</sup>). Patients were offered up to three attempts to eradicate HGD using ALA-PDT but in some cases this did not occur due to patient choice, technical reasons or the detection of cancer.

**Figure 8.4:** Diffuser fibre with aiming beam lit and inserted into an inflated DUSA balloon



**Follow up**

All patients remained in hospital following treatment with most discharged within 24-48 hours. Patients were discharged when eating and drinking and any chest discomfort controlled with mucogel or oral analgesia. Follow up endoscopies with quadrantic biopsies were taken every 2cm throughout the treated segment at 4 weeks, then every 3 months for the first year, every 6 months for the second year and yearly thereafter. The length and extent of the Barrett's segment was again formally mapped 6weeks-3months after PDT to assess squamous re-epithelialisation and compared to biopsies to ensure accuracy.

**Outcome measures**

The primary outcome measure was persistent or recurrent HGD. The reporting pathologist was blinded to the colour of activating light for all patients. The development of oesophageal adenocarcinoma and all side effects were also recorded.

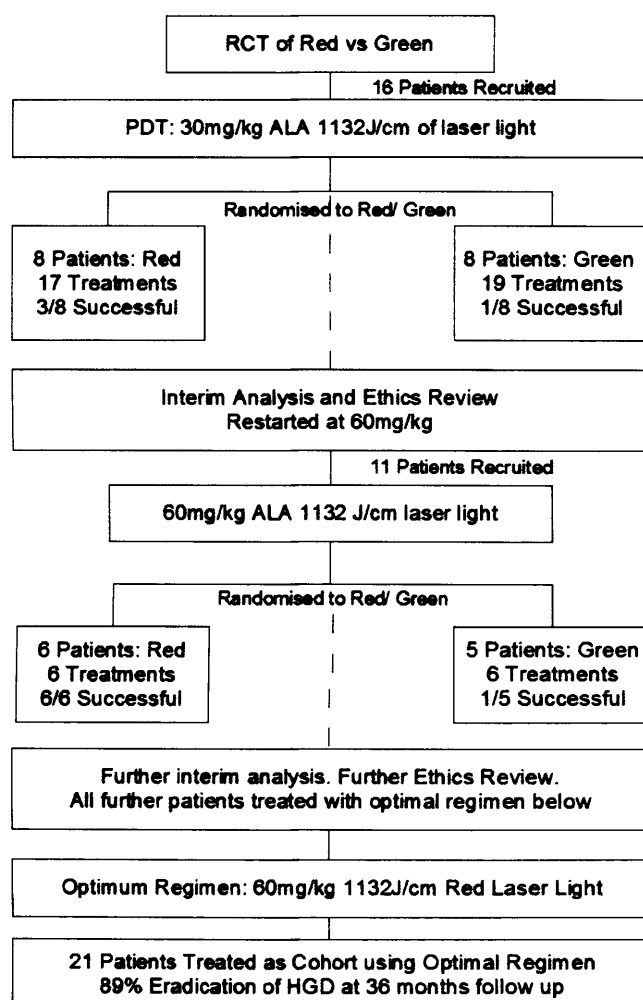
**Statistical Methods**

The patient groups were evaluated using an intention to treat analysis. Differences in pre-treatment parameters were compared using a t-test. Kaplan Meier curves were produced to compare length of time to relapse of HGD or cancer development. Log rank tests and Fisher's exact test were used to assess differences between the groups in univariate analysis and Cox's proportional hazards analysis used for multivariate testing.

### 8.3.3 Results

The study design, patient recruitment, PDT regimens and treatment results are shown in figure 8.5. The pre-treatment parameters of mean age, length of Barrett's and the number of levels of HGD were not significantly different between the treatment groups. (Table 8.6)

**Figure 8.5:** Flow chart of Patient recruitment and PDT Parameters



Successful PDT was defined as no recurrence of HGD and no cancer occurrence during the whole follow up period.

**Table 8.6:** Pre-treatment information by PDT Regimen (ALA Dose and Wavelength of Light)

PDT Regimen	n	Mean Age (Range)†	Mean Barrett's Length (Range)†	Mean Levels HGD (Range)†
60mg/kg ALA Red	27	70 (46-87)	7 cm (1-19)	1.9 (1-7 levels)
60mg/kg ALA Green	5	67 (46-80)	8 cm (3-11)	2.5 (1-6 levels)
30mg/kg ALA Red	8	70 (59-81)	7 cm (3-11)	2.1 (1-5 levels)
30mg/kg ALA Green	8	73 (61-80)	5 cm (3-9)	1.9 (1-3 levels)
<b>Overall</b>	<b>48</b>	<b>70 (46-87)</b>	<b>7 cm (1-19)</b>	<b>2.0 (1-7 levels)</b>

† No statistically significance difference

### Relapse to High Grade Dysplasia

An interim analysis was performed after 16 patients had been recruited, photosensitised and treated using 30mg/kg of ALA. Overall analysis suggested just 4 out of 16 patients had successful eradication of HGD (25%) with ALA at 30mg/kg. Success rate was only 13% (1/8) in the green group and 38% (3/8) in the red group. This compares to the expected success rates prior to the study of 95% for green light and 57% for red light. These results were discussed with the ethics committee and the study was stopped and restarted at 60mg/kg of ALA.

A further 11 patients were recruited and randomised to red or green laser light at 60mg/kg. Recruitment was stopped after the green laser broke irrevocably and a replacement laser was too expensive to purchase. All six patients treated at 60mg/kg ALA activated by red laser light had successful treatment compared to one out of five patients randomised to green laser light. These groups were significantly different in both a log rank test ( $p=0.008$ ) and in a Fishers exact test ( $p=0.01$ ). Table 8.7.

For confirmation of the earlier result that low dose ALA at 30mg/kg was less effective than 60mg/kg a subgroup analysis was performed. Patients treated with red laser light only were included and the different doses of ALA were compared. A log rank test and Fishers exact test were used and both displayed statistically significant differences with p values of 0.005 and 0.03 respectively. Table 8.7.

**Table 8.7:** Univariate analysis of RCT for the eradication of HGD using ALA by PDT Regimen

PDT Regimen	n	Developed Cancer	Relapsed to HGD/Cancer	Success Rate	Log Rank	Fisher's Exact Test
60mg/kg ALA Red	6	0	0	100%	NA Comparator	
60mg/kg ALA Green	5	1 (20%)	4	20%	$p=0.008$	$p=0.01$
30mg/kg ALA Red	8	1 (12%)	5	37%	$p=0.005$	$p=0.03$
30mg/kg ALA Green	8	4 (50%)	7	12%	$p<0.001$	$p=0.005$

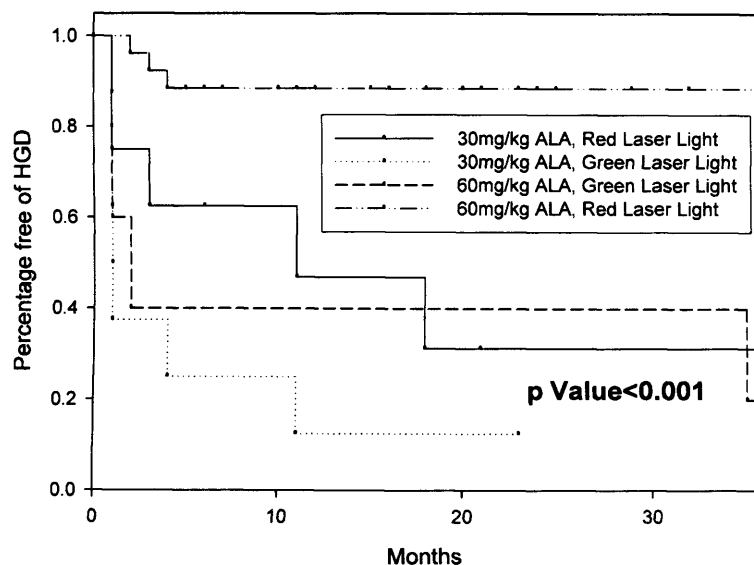
Levels of HGD; the number of 2cm levels of quadrant biopsies that showed HGD at baseline endoscopy

Twenty-one patients were then treated using the most effective regimen of ALA-PDT using 60mg/kg of ALA, activated by 1132J/cm of red laser light.

### Optimal Regimen Cohort

The estimated Kaplan Meier success rate of all the patients treated using the best regimen of ALA PDT in this study (n=27) was 89% at 36 months follow up. See figure 8.6 In a multivariate analysis comparing this regimen with lower dose ALA (30mg/kg) and colour of activating light (red or green) shows a significant difference between groups ( $p < 0.0001$ ). The hazard ratio calculated for relapsing to HGD when ALA 60mg/kg was compared to 30mg/kg was 0.30 (95% CI 0.11-0.74) and the hazard ratio for red rather than green light activation was 0.26 (95% CI 0.11-0.70).

**Figure 8.6:** Proportion of Patients free of HGD in RCT depending on regimen of ALA-PDT



Using 30mg/kg or 60mg/kg ALA activated by green light 60% of patients had persistent disease at first follow up. This compared to 25% of patients receiving 30mg/kg ALA activated with red light and 8% of those receiving 60mg/kg ALA activated by red light.

### Development of Adenocarcinoma

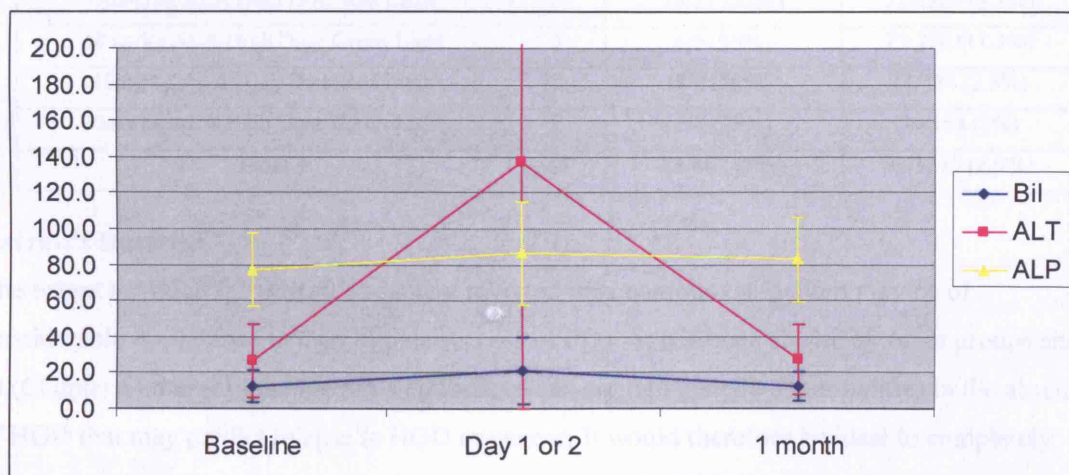
The overall cancer incidence with the optimal regimen was 4% during follow up. In a multivariate analysis red laser light was significantly better than green laser light for rate of future

cancer development ( $p<0.05$ ) but there was no significant difference in cancer rates between doses of ALA used (30 or 60 mg/kg). The study presented in section 8.2 above showed an increased rate of relapse to HGD with lower light doses with just 14% (2/14) of patients clear at 4 years follow up and the rate of cancer development with lower light doses was also significantly greater at 36% (5/14,  $p<0.01$ ).

### Side effects of ALA-PDT

One patient had a mild skin photosensitivity reaction with slight erythema of her face occurring 20 hours after receiving ALA 30mg/kg and no patients have developed oesophageal strictures. Two patients had self-limiting gastrointestinal bleeds one of which required a two unit blood transfusion. Both patients received the most effective regimen of ALA-PDT. Odynophagia occurred in 40% of patients following PDT but lasted only a few days. Nausea and/or vomiting started a few hours after treatment in just over half the patients but this stopped in all cases within 24 hours. Hypotension is a concern following administration of ALA at 60mg/kg but all serious hypotension has been avoided by intravenous hydration prior to the oral ingestion of ALA (Table 8.8). All patients receiving ALA at 60mg/kg had minor abnormalities in their liver function tests following drug administration but no patients had any long-term problems and all had returned to baseline levels by one month. (Figure 8.7) Four patients developed possible aspiration pneumonias after ALA-PDT but were treated with oral antibiotics and all recovered promptly. (Table 8.8)

**Figure 8.7:** Graph of mean and SD of average LFTs at Baseline, 1-2days and 1month post ALA-PDT



**Table 8.8: Summary of side effect profile of ALA by drug dose (red and green light)**

Dose of ALA	n	N&V	Hypo-tension	GI Bleed	Mild Photo-sensitivity	Chest Discomfort	Aspiration Pneumonia*
60mg/kg ALA	32	18 (56%)	2 (6%)	2 (6%)	0	18 (56%)	4 (12%)
30mg/kg ALA	16	10 (63%)	2 (11%)	0	1 (6%)	2 (12%)	0
<b>Total</b>	<b>48</b>	<b>28 (58%)</b>	<b>4 (8%)</b>	<b>2 (4%)</b>	<b>1 (2%)</b>	<b>20 (40%)</b>	<b>4 (8%)</b>

n, number of patients; N&V, Nausea and Vomiting;

\* Presumed aspiration pneumonia treated with oral antibiotics.

### Buried Glands

Buried Barrett's epithelial glands underneath regenerated squamous epithelium have been reported with all ablative therapies used in Barrett's oesophagus and occasionally, such glands can become dysplastic or even malignant (Van Laethem et al. 2000;Overholt et al. 2005;Van Hillegersberg et al. 2003;Overholt, Panjehpour and Halberg 2003). The percentage of the total number of follow up biopsies showing such glands was only 2.9% with the percentage falling to 2.1% in the group receiving the best PDT regimen. It is likely that the more biopsies are taken during follow up, the more buried glands that will be found. The overall per patient incidence of buried glands, however, in this study was 48%, which while higher than in some reports, is comparable to many other studies of ablative therapies including one study of Photofrin PDT (Basu et al. 2002;Ban et al. 2004;Ackroyd et al. 2004). The presence of buried glands was not associated with future relapse following PDT.

**Table 8.9: Buried Glands present in RCT depending on ALA-PDT regimen used**

PDT Regimen	n	Patients: Buried Glands Present	Biopsies with Buried Glands
60mg/kg ALA High Dose Red Light	27	14/27 (52%)	51/2380 (2.1%)
60mg/kg ALA High Dose Green Light	5	4/5 (80%)	23/208 (11.1%)
30mg/kg ALA High Dose Red Light	8	3/8 (38%)	11/484 (2.3%)
30mg/kg ALA High Dose Green Light	8	2/8 (25%)	10/168 (6%)
<b>Total</b>	<b>48</b>	<b>23/48 (48%)</b>	<b>95/3240 (2.9%)</b>

### Barrett's Reversal

The extent to which the Barrett's segment reverted to squamous epithelium may be of considerable importance to the ultimate success of PDT. It has been shown by other groups and us (Chapter 6) that residual Barrett's epithelium can contain genetic abnormalities in the absence of HGD that may predict relapse to HGD or cancer. It would therefore be ideal to completely reverse a patients Barrett's segment to squamous mucosa but this was not reliably possible using any regimen of ALA-PDT although the most effective regimen did on average result in more Barrett's reversal than the less effective regimens. See table 8.10. Additionally successful

eradication of HGD with PDT was associated with an average 50% Barrett's reversal compared to 5% with unsuccessful PDT (p value=0.0001 in a t test).

**Table 8.10: Percentage (to nearest 5%) of Barrett's reversal by regimen of ALA-PDT**

PDT Regimen	n	Mean % Reversal	p value (t test)
60mg/kg ALA High Dose Red Light	27	60%	Comparator
60mg/kg ALA High Dose Green Light	5	10%	p<0.001
30mg/kg ALA High Dose Red Light	8	-5%	p<0.001
30mg/kg ALA High Dose Green Light	8	0%	p<0.001

### Follow up endoscopy protocol

In the above study the patients received five surveillance endoscopies in the first year following PDT. Analysis of the timing of relapse to HGD or cancer in the optimal group suggested that the vast majority of patients (86%) fail treatment with incomplete eradication of HGD discovered at one of the first two follow up endoscopies. Only two other patients that relapsed to HGD occurred at 18 and 36 months which suggested that the number of endoscopies post PDT could be safely reduced to three in the first year at say 1month, 4months and then at one year. A similar pattern is seen for all treatment groups but those treated with lower light doses probably would require a more intense follow up if this were used in clinical practice due to a higher relapse rate overall.

**Table 8.11: Timing of relapse to HGD after ALA-PDT depending on regimen used**

PDT Regimen	n	Patients relapsed at 1 <sup>st</sup> /2 <sup>nd</sup> Endoscopy	Total relapses	Timing of other relapses
60mg/kg ALA High Dose Red Light	27	3 (100%)	3	NA
60mg/kg ALA High Dose Green Light	5	3 (75%)	4	36m
30mg/kg ALA High Dose Red Light	8	3 (60%)	5	12m, 18m
30mg/kg ALA High Dose Green Light	8	6 (86%)	7	12m
<b>Total</b>	<b>48</b>	<b>15 (79)</b>	<b>19</b>	<b>12m, 12m, 18m 36m</b>



#### **8.3.4 Discussion**

Many different regimens of ALA PDT have been suggested to treat dysplasia in BO. The amount of ALA used for photosensitization has varied from 30-60mg/kg and the amount of light used for activation varied from 264-1000J/cm length of diffuser fiber. Success rates between 17% and 89% have been reported depending on the dose of drug and light used for the eradication of HGD in BE (Forcione et al. 2004;Pech et al. 2005;Peters et al. 2005b). The largest of these studies showed a good eradication rate for HGD (31/35 patients, 89%, at 37 months follow up) with ALA 60mg/kg and a light dose of 150J/cm<sup>2</sup> (the balloon size not reported but is believed to be 18mm which would translate to approximately 900J/cm diffuser fiber length) (Pech et al. 2005).

In two studies treating low-grade dysplasia excellent eradication rates were achieved using green light at much lower light (264J/cm) and drug (30mg/kg) doses (Ackroyd et al. 2003;Ackroyd et al. 2000b). All of the above studies have only examined a single set of parameters for ALA-PDT. Green light might have been expected to be more effective in the eradication of HGD as it has been successful using far less aggressive PDT parameters for the treatment of LGD (Ackroyd et al. 2003;Ackroyd et al. 2000b). The extinction coefficient of PPIX has been shown to be higher at 512nm than at 635nm and therefore for equal light doses green light might also be expected to perform better but this was not seen in our study.

All these studies have used different criteria for inclusion, different cohorts of patients and different treatment parameters. This makes comparisons of the relative efficacy of PDT difficult. Until now, the most effective parameters of ALA PDT have not been known. Recently we have published data concerning light dose showing that 1000J/cm is more effective than lower light doses (Mackenzie et al. 2007) and the data in the current study help to resolve these issues further for the treatment of HGD. This study therefore supports 60mg/kg ALA activated by 1132J/cm of red laser light as the optimal treatment regimen with an 89% eradication rate for HGD. The Kaplan Meier estimation of long-term cancer incidence with the optimal regimen was 4% at 3 years follow up. Although this study was stopped early for technical reasons with a smaller number of patients recruited than planned the data was still statistically significant even after correction for these smaller numbers in a Fisher's exact test.

Some questions over the most effective regimen of ALA-PDT do remain. Two other, albeit less studied, parameters exist that might influence the success of treatment; the fluence rate at which laser light is delivered and whether the light dose is fractionated. There is some evidence that suggests the lower the fluence rate the more effective the PDT although the relationship is complex due to light scattering within the oesophagus. Ultimately, it may be necessary to measure fluence rates in real-time, *in vivo*, in order to understand this relationship clearly (Boere et al. 2006). Irrespective of this the laser time is already 35 minutes with a fluence rate of 565mW/cm of diffuser fiber delivered in an 18mm balloon (100mW/cm<sup>2</sup>) and if two segments need to be treated then the total procedure time rises to an hour and 15 minutes. Any reduction in the fluence rate would require a similar proportional reduction in laser light necessary for effective treatment in order not to prolong the procedure. This is important both for reasons of patient comfort and risk of aspiration pneumonia. It is not practical to perform ALA PDT with the patient fully awake as the procedure is painful for the patient. The second potential methodological change is light dose fractionation. Some studies have suggested it could improve efficacy both in animals (Curnow et al. 1999; Pech et al. 2002) and in humans (Hage et al. 2004) but there is no large clinical study supporting its use. Its effectiveness and safety in humans, therefore, remains unproven.

Photofrin has led the way in PDT for HGD in BO and it has been shown to reduce cancer rates by 50% (Overholt et al. 2005). These results have allowed Photofrin to gain a license for the treatment of this condition and, at present, it is probably the minimally invasive therapy of choice for diffuse, flat HGD. The side effects of Photofrin PDT, however, can be difficult with prolonged skin photosensitivity and 22-50% oesophageal stricture rate depending on the number of treatments received.

ALA-PDT avoids the complications of Photofrin PDT. It appears to be safer, but is not entirely risk free. More than 300 ALA treatments have been reported worldwide (Gossner et al. 1998; Ackroyd et al. 2000a; Ackroyd et al. 2000b; Forcione et al. 2004; Hage et al. 2004; Kelty et al. 2004a; Kelty et al. 2004b; Pech et al. 2005; Peters et al. 2005a; Mackenzie et al. 2007) and two patients have died shortly after ALA administration (Forcione et al. 2004; Hage et al. 2004). It is unclear why these events occurred but one autopsy reported aspiration pneumonia as the cause of death and both cases were treated as outpatients. Eight percent of patients in this study developed some clinical signs of aspiration pneumonia following therapy and were treated with oral antibiotics. All episodes resolved promptly but patients routinely remained in hospital for 24-48

hours following PDT. We have also noted mild hypotension but serious episodes were successfully averted by giving intravenous fluids prior to ALA administration. Although in this study IV fluids were given 8 hours prior to drug ingestion there is no clear evidence what the necessary time frame for rehydration is but because of this hypotensive risk all patients were admitted to hospital 24 hours prior to treatment and they remained in hospital for a minimum of 48 hours.

There has been a single case report of a patient who developed a severe neuropathy after ALA administration but we have not seen this in any of our patients (Sylantiev et al. 2005).

Buried glands are a concern following PDT as these columnar cells can become malignant and subsquamous carcinomas have been reported (Overholt et al. 2005; Van Laethem et al. 2000). Furthermore surveillance is difficult as these cells are not visible to the endoscopists and therefore their detection is reliant on random biopsy. In this cohort the overall percentage of buried glands is 3.5% of biopsies and 2.6% in the optimal regimen group which is a similar rate to other studies of ablative therapies. Fifty-one percent of patients however had buried glands found at some point after treatment although 78% (14/18) of these patients in the optimal regimen group had these glands found only once. This overall rate of buried glands (51% of patients) is higher than in some reports but is comparable to many others including one study of Photofrin PDT (Basu et al. 2002; Ban et al. 2004; Ackroyd et al. 2004). The high rates of buried glands in our study may be due to the consistent use of large cup biopsy forceps which have not been used by all other groups. Larger biopsy samples may lead to more buried glands being identified. What does seem to be the case is the likelihood that more buried glands that will be found with the more biopsies are taken during follow up.

Complete Barrett's reversal would be an ideal goal for any ablative therapy hence by definition removing all residual genetic abnormalities. This has not been realistically possible for most patients with any ablative therapy until recently and in this study the best regimen of ALA-PDT only reversed on average 60% of a patients Barrett's. Follow up is currently therefore an enormous burden for all ablative therapies as well as in surveillance for BO. As few as 4% may develop cancer within three years of therapy but these patients will have been subjected to an average of 8 endoscopies in this timeframe. Endoscopic follow up is necessary at present on two counts; the first being the high prevalence of buried glands and the second being the incomplete reversal of the Barrett's segment with the consequently the risk of harboured genetic

abnormalities. The data above, however, suggests that five endoscopies in the first year after treatment are unnecessary and excessive. If two endoscopies were performed in the first 4 months after treatment then this would have detected all relapses to HGD or cancer in the group receiving the best regimen. This could be followed by a further endoscopy at one year then at 18 months and 2 years followed by yearly endoscopies thereafter. This at least reduces the post PDT follow up by 2 endoscopies to 6 in three years. This might be reduced further to just two endoscopies after PDT running the second biopsy series for aneuploidy if the results in Chapter 5.5 are confirmed with a third at four or five years. The measurement of aneuploidy for this purpose requires testing in a larger study but offers a promising alternative to such regular endoscopies. This reduction of procedures would significantly lower the burden of follow up for both the patient and the clinician.

For the first time, however, these problems might be avoided using the new radiofrequency ablation technique HALO<sup>360</sup>. Early data suggests good complete eradication rates for Barrett's (up to 95%) but no long term follow up (>12 months) has been reported and no data exists about the rates at which Barrett's might recur (Sharma et al. 2007; Roorda, Marcus, and Triadafilopoulos 2007). Several authors have reported Barrett's recurrence following oesophagectomy (Hamilton and Yardley 1977; Oberg et al. 2002; Lindahl et al. 1990) with a more recent study of showing that 8/36 (22%) patients who have had subsequent endoscopies post surgery have been found to have further BO (Wolfsen, Hemminger, and DeVault 2004). The authors of this study attribute Barrett's recurrence to uncontrolled acid reflux despite post-operative proton pump inhibition. Although acid reflux is more likely in this group, this does raise the question of recurrent BO forming after previous complete ablation with HALO<sup>360</sup>. Furthermore, although buried glands are rare with radiofrequency ablation a case report has now been published demonstrating buried glands post radiofrequency ablation (Mashimo et al. 2007) even using the more strict definition used by the commercial company BarrX than those used in most other studies of ablative therapy (Bergman 2007).

Comparative randomised controlled trials are required to compare the leading minimally invasive treatments for HGD in Barrett's Oesophagus. These results would suggest a trial to compare ALA (60mg/kg) activated by at least 1132J/cm of red laser light with Photofrin PDT, currently the minimally invasive treatment of choice. Prior to the early data and commercial release of radiofrequency ablation with HALO<sup>360</sup>, a randomised controlled trial was, therefore, designed to compare ALA against Photofrin PDT, the minimally invasive treatment of choice at the time.

## **8.4 A randomised controlled trial of Photofrin versus 5-aminolaevulinic acid photodynamic therapy in the eradication of high grade dysplasia in Barrett's Oesophagus**

### **8.4.1 Introduction**

The previous chapter resolves many of the outstanding parameter questions associated with ALA-PDT. The regimen of ALA-PDT that gives the best rate of HGD ablation in Barrett's oesophagus appears to be 60mg/kg of ALA activated by 1132J/cm of red laser light.

The National Institute for Clinical Excellence (NICE) has recently approved Photofrin Photodynamic therapy for treating HGD in BO. Photofrin has led the way in minimally invasive therapy for flat HGD in BO and should therefore be considered at present the minimal invasive therapy of choice. The RCT of Photofrin PDT plus PPI compared to PPI alone in showing a 50% reduction in cancer risk has placed a marker in the ground for all other ablative therapies to be compared against (Overholt et al. 2005).

Pech et al following the publication of their cohort of patients treated with ALA-PDT recommended "*A prospective randomised trial is needed to clarify the relative toxicities of these two porphyrins*" (Pech et al. 2005).

For these two reasons Photofrin PDT was chosen as the control arm of this partially blinded RCT comparing the safety and efficacy of ALA and Photofrin PDT for the eradication of HGD in BO.

### **8.4.2 Aims**

The aim of this single centre RCT was to determine whether PDT for HGD in BO using ALA is more effective than Photofrin and if ALA has less side effects.

### 8.4.3 Methods of RCT of Photofrin versus ALA-PDT

#### Power Calculations

In order to show a 20% difference in the development of oesophageal strictures (presuming 1% for ALA and 21% for Photofrin) and cutaneous photosensitivity reactions (5% for ALA and 25% for Photofrin) between the 2 treatment protocols at a 1-sided significance level of  $p < 0.05$  and a power of 80%, 33 patients are required in each group allowing for a 10% drop out rate.

From our previous work the overall 5 year eradication rate of HGD with ALA-PDT is approximately 80% compared to 50% for Photofrin in the RCT PDT and PPI versus PPI alone. In order to show this difference in the eradication rates for HGD of 30% at significance level of 0.05 in a 2-sided test with a power of 80%, 33 patients are also required in each group (allowing for a 10% drop out rate).

#### Regulatory Approvals

This study was sponsored by University College London. Ethics approval was received from Berkshire Research Ethics Committee (COREC Number 05/Q1602/193) and the study was approved by the Medicine and Healthcare Regulatory Authority (MHRA) as the competent authority for the UK (Eudract Number 2005-005528-15). This study has been registered with ISRCTN, International Standard Randomised Controlled Trial Number Register (ISRCTN 16444200).

**Table 8.12:** Inclusion and Exclusion Criteria for RCT of ALA versus Photofrin PDT

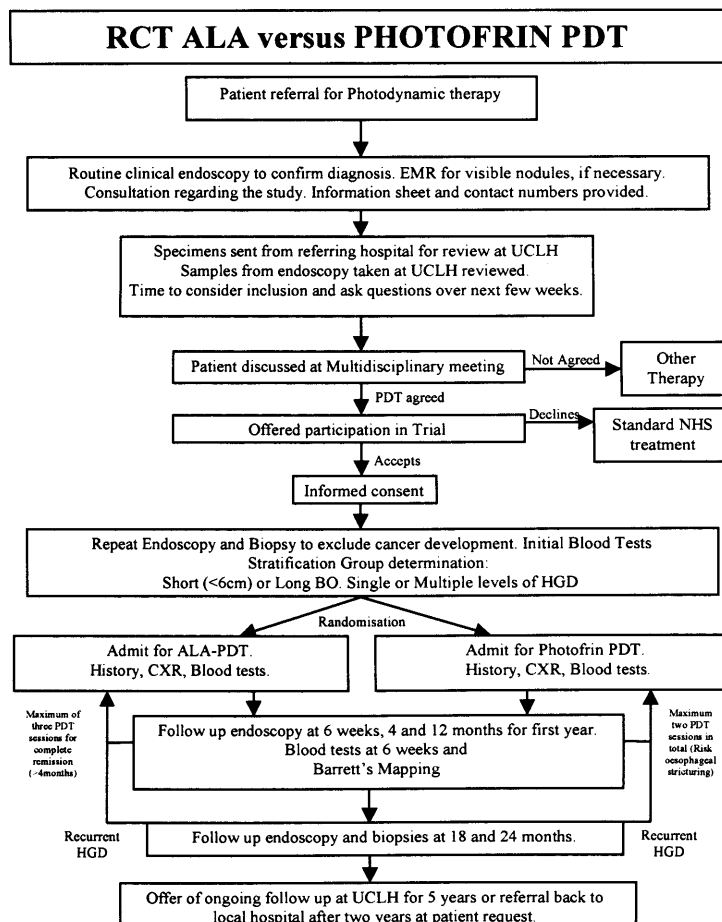
Inclusion	Exclusion
Patients with HGD in BO confirmed by 2 independent pathologists	History of severe cardiovascular disease, severe angina, congestive heart failure, or recent syncope of cardiovascular origin
Following EMR to visible distinct nodule of HGD with residual HGD	Patients presenting with abnormal cardiac signs or symptoms
Normal EUS	Evidence of invasive cancer
No contraindications to endoscopy	WBC $< 2 \times 10^9/L$ , Platelet count $< 50 \times 10^9/L$ ,
Males and non-pregnant females over the age of 21 years	Impaired renal function at time of entry into the study (Creatinine $> 200 \text{ mcml/L}$ )
Patients able to give informed consent	Impaired hepatic function (Bilirubin $> 50 \text{ mcml/L}$ , ALP or ALT $> 2$ times upper limit of normal)
BO no greater than 13cm long	Prothrombin time $> 1.5$ times the upper limit of normal
No prior PDT for Barrett's Oesophagus	Patients who have a history of porphyria
No evidence of invasive cancer on two endoscopies and quadrant biopsies	Patients receiving chemo- or radiotherapy or chemotherapy within 4 weeks of entry into this study
	Patients on depot preparations of psychotropic medication
	Patients with orthostatic hypotension resistant to hydration

### **Trial Protocol** (See figure 8.8 for flowchart)

Prior to enrolment into the study once a referral was received the patient had a routine endoscopy arranged as usual in order to confirm the diagnosis of HGD and to ensure that the presence of cancer was excluded. An EUS was performed and any nodules found were removed by EMR. Quadrantic biopsies every 2cm of Barrett's Oesophagus was taken for routine histology and were reviewed by an expert gastrointestinal pathologist. Additionally, slides collected at the referring hospital were requested and reviewed by this pathologist to confirm the original diagnosis.

During this visit a consultation was held and the patient informed about this RCT. The study information sheet was provided and initial questions answered. The patient's history and biopsies were reviewed at the upper gastrointestinal multidisciplinary meeting and if appropriate the patient was then offered participation in the clinical study. A further interview was arranged to discuss the trial, any further questions were answered and then informed consent was taken. The process of informed consent occurred throughout this referral and assessment period (approximately 2-8 weeks in total).

**Figure 8.8: Protocol Flow Chart for RCT of ALA versus Photofrin PDT**



### **Following enrolment in the study**

**PDT planning endoscopy:** A repeat endoscopy and quadrantic biopsies every 2cm were performed to reduce the chance of missed oesophageal cancer and to plan the PDT treatment.

**Randomisation:** Following double stratification, to long (>6cm) or short segment Barrett's oesophagus and single or multiple 2cm biopsy levels of HGD, patients were randomised to receive either ALA or Photofrin-PDT.

**Prior to PDT:** A full history and physical examination were carried out with particular focus on symptoms of oesophageal reflux, lifestyle and previous medication. Additionally, a chest x-ray, ECG, full blood count, urea and electrolytes and liver function tests were also performed. Patients were prescribed Omeprazole 40mg or equivalent daily prior to PDT.

### **Methodology of ALA-PDT**

**Oral ALA administration:** ALA (99% purity) was supplied by DUSA Pharmaceuticals, New York, USA and manufactured to the standard of Good Manufacturing Practice. ALA was weighed and dispensed from the UCLH Pharmacy. A normal saline infusion was started at least 12 hours prior to oral administration of ALA and intravenous Granisetron (2mg) was given 30 minutes before. ALA was given in three divided doses of 20mg/kg each dissolved in 50mls of distilled water 5, 4, and 3 hours prior to PDT. Patients were advised to avoid strong indoor lighting and sunlight for 36 hours.

**Light Delivery:** Light delivery is via a diffuser fibre placed centrally in an 18mm diameter DUSA balloon (figure 8.5 above) and the treated area included at least 0.5cm of normal tissue treated at the margins of the treatment section. The maximum length of BO with HGD that may be treated at one session was 13cm.

For ALA-PDT light was delivered via a diffuser fibre at 1132J/cm at 635nm (or 200J/cm<sup>2</sup> in an 18mm diameter balloon) with a fluence rate of 565mW/cm of diffuser fiber (100mW/cm<sup>2</sup>).

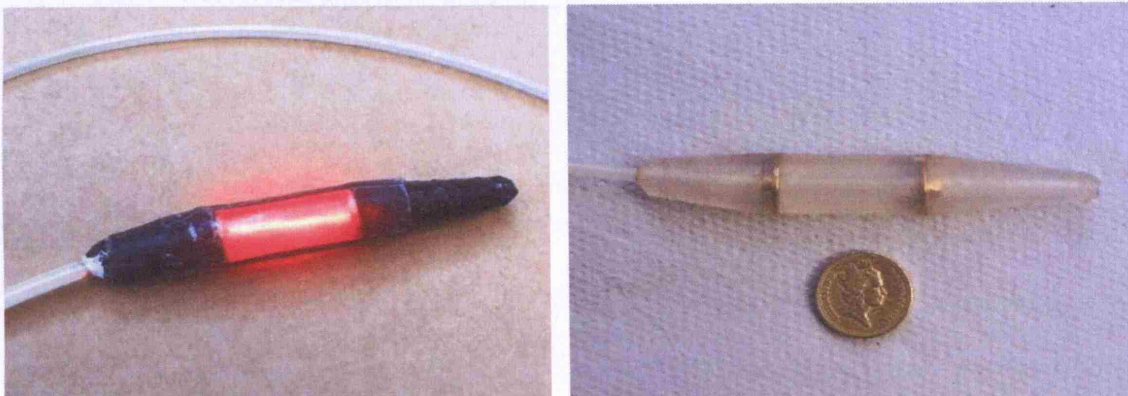


**Methodology of Photofrin PDT:** Patients were treated according to the SmPC supplied by Axcan and the papers published in this area (Panjehpour and Overholt 2006;Overholt et al. 2005). Photofrin was given intravenously at 2mg/kg 48-72 hours prior to light activation. Following administration of Photofrin, patients were counselled to minimise direct exposure to sunlight and avoid strong indoor lighting. A light metre was provided as well as demonstration of its use and written light precautions supplied.

During PDT the same Seldinger technique described above for ALA was used for insertion of an 18mm silicone bolster as opposed to a DUSA balloon. Figure 8.9. No overlap between the treated segments was permitted however with Photofrin due to the risk of oesophageal stricture formation. A light dose of 130J/cm at 630nm was used with a fluence rate of 250mW/cm.

Patients were allowed two attempts with Photofrin PDT to eradicate their HGD in the RCT below due to the very high (50%) risk of oesophageal stricturing with three attempts. Additionally, a time period of three months between the administrations of Photofrin was necessary to avoid cumulative skin photosensitivity.

**Figure 8.9:** Silicone bolster used for Photofrin PDT



**Follow up for all patients:** During the first three days after treatment the patients had their liver function tests measured at day 1 or 2 and repeated if significantly different from baseline. A repeat chest X-ray was taken prior to discharge if clinically indicated. The post-treatment follow-up protocol was regular endoscopic evaluation of the PDT response at 6 weeks and quadrantic biopsies every 2cm of previously treated mucosa were taken. All patients had liver function tests repeated at six weeks after therapy.

Patients then had a surveillance endoscopy and quadrantic biopsies every 2cm of the previously treated area at 4, 12, 18 and 24 months and then yearly thereafter. The total envisaged patient follow up was at least 5 years. All biopsies were reported by the pathologists blinded to the type of PDT performed and all relapses to HGD confirmed by two independent pathologists.

#### **End Points for the study**

**Table 8.13:** The clinical endpoints of the RCT of ALA versus Photofrin PDT

<b>Primary End Points</b>	<b>Secondary End Points</b>
Eradication of HGD	Comparison of all other side effects
Prevention of cancer development	Presence of buried glands
Rates of oesophageal stricture formation	Area of Barrett's mucosa re-epithialised with squamous mucosa
Rates of cutaneous photosensitivity	

#### 8.4.4 Results

Forty-four patients were enrolled into this trial up to 1<sup>st</sup> October 2007. Four patients were screened out (one had intramucosal cancer on initial study endoscopy, one was taking depot antipsychotic, one had active congestive cardiac failure and one patient withdrew). Thirty-two patients, approximately half of the total planned to be recruited (total number 66), have received PDT. Sixteen of these 32 patients have been randomised to each group for PDT. As expected with stratification prior to randomisation the mean length and 2cm biopsy levels of HGD are similar between the two groups. Five patients are undergoing repeat therapy, 3 with photofrin and 2 with ALA leaving 27 that have reached 4 months follow up or reached a primary endpoint of the study.

**Table 8.14:** Pre-PDT patient parameters for those treated to date in RCT of ALA versus Photofrin PDT

PDT Group	Randomisation Group					Age (Range)	Mean Length BO (Range)	Mean Lvl's HGD (Range)
	N	SS	SM	LS	LM			
ALA	16	5	4	1	6	68 (49-83)	6.5 (1-13)	2.1 (1-5)
Photofrin	16	6	3	0	7	67 (45-82)	6.6 (3-13)	2.1 (1-4)
<b>Total</b>	<b>32</b>	<b>11</b>	<b>7</b>	<b>1</b>	<b>13</b>	<b>68 (45-83)</b>	<b>6.6 (1-13)</b>	<b>2.1 (1-5)</b>

SS: Short BO, Single level of HGD; SM: Short BO, Multiple HGD; LS: Long BO, Single level of HGD; LM: Long BO, Multiple levels HGD.

#### **Eradication of HGD following randomisation to ALA or Photofrin PDT**

Of the 32 patients randomised and treated to date the 16 patients treated with Photofrin have required 23 treatment and the 16 patients treated with ALA have required 25 treatments. The eradication rates of ALA-PDT appear to be better than those for Photofrin PDT but the numbers are small and the follow up short (minimum 4 months, two clear endoscopies).

**Table 8.15:** Eradication Rates of HGD at 4 month follow up for ALA and Photofrin PDT

PDT Group	n	Success †	Failure †
ALA	14	14	0
Photofrin	13	8	5
<b>Total</b>	<b>27</b>	<b>22</b>	<b>5</b>

† Fishers Exact Test p Value <0.05

## Side effect profile

**Table 8.16:** Side effect profile of ALA and Photofrin PDT

PDT Group	n	Low BP	Pleural effusion	N&V	Strictures	Photo-sensitivity	Chest Discomfort	Aspiration Pneumonia
ALA	16	1	2	7	1	0	4	1
Photofrin	16	0	2	5	6	6	3	2
<b>Total</b>	<b>32</b>	<b>1 ♦</b>	<b>4 ♦</b>	<b>12 ♦</b>	<b>7 †</b>	<b>6 †</b>	<b>7 ♦</b>	<b>3 ♦</b>

† p value<0.05 in single sided Fishers Exact Test

♦ p value non-significant in Fishers Exact Test

To date, 7 serious adverse events (SAEs) have occurred. An SAE is defined under European Union (EU) regulations and those relevant to this trial as an event that is either life threatening, results in death, prolongs the length of inpatient stay or that requires admission to hospital. Five of these events have occurred in the Photofrin arm and 2 in the ALA arm.

With respect to Photofrin, one patient suffered from severe chest discomfort requiring admission to hospital, one patient experienced severe skin photosensitivity and one developed a pleural effusion that required drainage due to breathlessness resulting in a prolonged length of stay. Two patients have developed post-PDT pneumonias lengthening their inpatient stay and one of these has subsequently died from an episode of antibiotic related Pseudomembranous Colitis, which occurred 4 days after discharge from hospital following PDT. The patient did not develop diarrhoea until after discharge from hospital.

Two SAEs have been reported from the ALA group. The first was a para-tracheal neck abscess occurring 4 months after therapy. The opinion of the external ENT surgeon was that it was not related to this patient's PDT, it has been drained and the patient is well. More seriously one patient post ALA-PDT developed an acute coronary syndrome on day 2. This was reported, by the cardiologists, to have occurred due to a complete occlusion of the right coronary artery. This patient also had an additional 50% chronic stenosis of the left anterior descending artery. The right coronary artery was stented and re-opened, the patient was discharged, and went home well. Although severe cardiac disease is an exclusion criterion this patient was asymptomatic with a normal ECG at enrolment and it would appear, therefore, that ALA-PDT unmasked silent ischaemic heart disease.

There are already statistically significant differences in the oesophageal stricture rates and frequency of light photosensitivity reactions (Fishers Exact test<0.05, table 8.16). The median number of oesophageal dilatations required in the photofrin group was 3 and the single stricture in the ALA group required one dilatation. The patient in the ALA arm that develop an oesophageal stricture had significant acid reflux for 60% of the period of monitoring on her 24hr oesophageal manometry despite high dose PPI (Omeprazole 40mg BD). This stricture was likely to be related to extremely high levels of acid reflux on top of a mucosal oesophageal injury rather than directly from ALA PDT injury. One of the patients who developed an oesophageal stricture in the photofrin arm of the study had received previous radiotherapy 6 years prior to PDT. In hindsight, although not a contraindication to photofrin PDT, this previous radiotherapy probably increased his oesophageal stricture risk.

Nausea and vomiting is common after both ALA and Photofrin PDT but slightly more frequent in the ALA group. Chest discomfort occurred with similar frequency in both groups at 25% (4/16) in the ALA group and 18% (3/17) in the photofrin group. Liver function tests became mildly abnormal in 75% (12/16 patients) in the ALA group and in 29% (5/17) in the Photofrin group but all had returned to normal by first follow up. A summary of all the common side effects are listed in table 8.16 above.

#### **8.4.5 Discussion**

There appears to be an early divergence of the success rates of ALA and Photofrin PDT with approximately one third of those patients who received Photofrin failing treatment compared to none to date who received ALA. The follow up to date however is short with only 27 patients having reached 4months follow up. One reason for this apparent difference in success rates might be that the protocol of this study allowed three treatments with ALA but only allowed two treatments with Photofrin. In the RCT of Photofrin PDT plus PPI versus PPI alone three treatments were allowed. Not offering a third treatment may have adversely affected the success rate of Photofrin PDT. At inception, it was felt a 50% risk of oesophageal stricture formation was too high a risk and potentially unethical in a RCT with one therapy having extremely low risk of stricture formation. As stricture formation is also one of the primary endpoints in this study, three repetitive treatments with Photofrin could also be criticised as potentially biasing the safety data of the two drugs. Ultimately the decision was made to limit the treatment protocol with Photofrin to two treatments, the external reviewer of the protocol prior to this trial starting did not highlight

this as a major weakness and the ethics committee felt this to be the appropriate number of treatments given the data available. Despite this there is already a statistically significant increase in the rates of oesophageal stricture formation and skin photosensitivity with the photofrin PDT in a one sided Fishers exact test.

The follow up of patients is very short and all interpretation needs be cautious and bear this fact in mind. The data in sections 8.2 and 8.3, however, would suggest that very few patients if treated with the best regimen of ALA-PDT relapse after 2 clear endoscopies (1/38 from the two studies above). The study has to be completed and follow up for at least two years necessary to fully interpret these results but this interim analysis is encouraging.

It would also appear that ALA-PDT is safer than Photofrin. There have been fewer SAE, fewer photosensitivity reactions and fewer oesophageal strictures in the ALA group. This trial is on-going.

## **8.5 Summary**

In this chapter evidence is presented to support the best regimen of ALA-PDT being of 60mg/kg ALA activated by 1132J/cm of red laser light. This does not account for all the possible variables relating to PDT with no data addressing light dose fractionation and the fluence rate of the laser power used. These two are less studied parameters, however, and the variables for clinical testing less well defined. The additional study of these two parameters from a practical perspective within a single unit poses an almost insurmountable problem with respect to the number of parameters being studied and the number of patients available to be enrolled. Irrespective of this, an eradication rate of approximately 90% for ALA-PDT does warrant proper testing against the other current leading minimally invasive treatments for HGD in Barrett's Oesophagus. Even Photofrin PDT, which has lead the way for the ablation of flat diffuse HGD, has been subject to continued post marketing studies in particular examining light dose in an attempt to reduce stricture formation. Indeed, those involved with the RCT of Photofrin plus PPI compared to PPI alone will admit that one of the principle aims of that study was to essentially place a 'marker in the ground' as a standard for all other ablative therapies to at least match.

The early data from the RCT of ALA versus Photofrin is encouraging for ALA from both the perspectives of safety and efficacy but longer follow up and more patient recruitment are required.

Like diagnostic endoscopy, this is a rapidly moving field and new therapies are continuously becoming available. In the last three years new methods including extensive EMR with a multiple banding technique, balloon based radiofrequency ablation (HALO<sup>360</sup>) and most recently at Digestive Disorders Week 2007 cryotherapy was presented. Of these the simplest to use, with the most supporting data, and most freely available is radiofrequency ablation. This would, therefore, seem to be the most immediately apparent choice for further study. Radiofrequency ablation has several advantages in that it offers potentially complete BO eradication, has excellent short term (up to 12 months) clearance rates for HGD of 85-95%, does not appear to leave buried glands and is a day case procedure. Radiofrequency ablation, however, does require more endoscopies with a median of 1.4 sessions with HALO<sup>360</sup> and 2.5 additional sessions with HALO<sup>90</sup>. Were the early data in the RCT between ALA-PDT and Photofrin to be confirmed then ALA-PDT compared to radiofrequency ablation for the treatment of HGD in BO would be an area for future study.

## Chapter 9

# Conclusions and the Future



## **9 Conclusions and the Future**

### **9.1 Aims of the thesis**

The principle problems with surveillance and therapy of Barrett's Oesophagus that are addressed in this thesis are:

1. How can we more accurately define a high risk group in the surveillance population?
2. Can we assist the detection of this high risk group in surveillance by the novel diagnostic technology of ESS?
3. Having defined this high risk group are there minimally invasive therapies that are effective but carry lower risks than oesophagectomy?

In answer to the first point the problems with histology are well documented. In summary they surround the lack of agreement regarding dysplasia between pathologists, the random nature of lesion detection even with a predefined systematic (but essentially random) biopsy protocol and the poor predictive ability of HGD. If it were possible to reliably measure aneuploidy, in addition to HGD, this might offer significant improvement in particular to freeing up over 85% of patients from follow up for at least five years. Even were aneuploidy to be measured reliably this still is not the whole solution because while effective at stratifying low risk patients its predictive ability for future cancer risk is still only 28% without HGD and 66% with HGD. The temporal relationship of aneuploidy to future cancer risk, however, appears to be reasonable. This offers both the patient and the clinician a reasonable time frame to eradicate the lesion prior to cancer development.

The biggest single barrier to the implementation of aneuploidy in routine clinical practice to date however has been the difficulty of sample collection by cooling and then freezing prior to applying fastidious methodology in the laboratory to avoid errors resulting in false positive histograms. This methodology has prevented the use of flow cytometry for the detection of aneuploidy outside one or two research laboratories.

Unfortunately, this is not the only issue with the detection of HGD and aneuploidy. The detection of these abnormalities is also expensive and labour intensive. This thesis has shown that even amongst those patients with HGD (approximately 2.5% of a surveillance population) only 1 in every 8 biopsies taken contains HGD. While this is better for the detection of aneuploidy on a posterior biopsy series at approximately 1 in 12 surveillance biopsies this is probably still too

expensive. Indeed the value of surveillance is being currently questioned by the NHS, amongst others, and a RCT of surveillance versus no surveillance is now recruiting. Ultimately, standard white light endoscopy with quadrantic biopsy every 2cm is almost certainly not going to be accurate enough but the breathtaking developments in novel diagnostics suggest that this might not be the case in five years time. The real challenges for these diagnostic technologies are to be cheap, fast, compatible with existing endoscopes, with little or no interobserver variation and extended periods of specialist training not required. ESS fulfils many of these basic criteria but the question of algorithm development and ongoing accuracy needs to be assessed.

Finally, when starting my studies the only widely available therapy for HGD in Barrett's was oesophagectomy with all of its adherent risks. Photofrin PDT was under assessment in a RCT to compare its effectiveness to proton pump inhibition alone but even this potentially promising therapy had significant side effects of prolonged skin photosensitivity and oesophageal stricture formation. Other therapies such as EMR appeared effective for nodular disease but were impractical for widespread flat HGD, and APC and thermal laser probably less effective than PDT. There was not even a whisper of radiofrequency ablation with HALO<sup>360</sup> for HGD in Barrett's Oesophagus.

The third aim, therefore, was to work with ALA PDT to develop, within practicality, the optimum parameters for its use and then compare these parameters to Photofrin which has been the market leader of minimally invasive therapies for flat diffuse HGD in Barrett's Oesophagus. Photodynamic therapy offered patients for the first time a real alternative to oesophagectomy or surveillance for HGD in BO. It is clear now that ablative therapies in Barrett's are moving extremely rapidly with exciting new therapies emerging each year. In May 2006 the new technique was radiofrequency ablation and in May 2007 it was cryotherapy. One cannot help but wonder about this years entries. This rapid and novel technological advance in upper GI therapeutics is likely to set a trend for endoscopic therapy across the whole GI tract.

#### ***9.1.1 Validation of image cytometry to detect aneuploidy in BO***

**The results in this thesis:** Flow cytometry, as opposed to image cytometry, is the accepted gold standard for detecting aneuploidy in Barrett's Oesophagus. The methodology for sample processing is difficult with only a few research laboratories able to accurately reproduce results. Secondly the specimens at present have to be fresh frozen during the endoscopy procedure

essentially making it very difficult to collect samples from outside the direct region of the research facility.

Identifying aneuploidy using image cytometry via the methodology presented in this thesis potentially circumvents these two particular problems. Using cut sections from paraffin embedded tissue allows not only archive specimens to be processed but specimens collected during routine endoscopy can be transported far more easily and processed centrally. Additionally, the methodological issues regarding clumping and cut nuclei are less with image cytometry as nuclei are viewed prior to analysis and these nuclei are removed. In this thesis a methodology was developed for processing paraffin embedded tissue and in a small sample image cytometry was shown to produce very similar results to flow cytometry performed in Seattle.

Some new errors are potentially introduced by the methodology developed and these need to be examined, understood and estimated for. Although smaller numbers of nuclei can be processed for aneuploidy estimation accurate detection of tetraploidy requires at least 600 nuclei to be collected and even then a small reduction in the certainty of the estimate of the 4N fraction exists. The last significant error that may be produced is by cutting a 40micrometre section from a histology block. Aneuploid and tetraploid nuclei are larger than diploid nuclei and therefore have a greater chance of being cut. This alteration in chance will affect the proportions in the aneuploid or tetraploid peaks thereby reducing the optimum cut-off for image abnormalities. These errors were estimated for and appear to be relatively small. The final definitions decided upon for this methodology were:

***Aneuploidy:** a second peak of greater than 2.3% of the nuclei in a histogram not between 3.85N and 4.1N.*

***Tetraploidy:** considered present if the 4N peak (3.85-4.1N) consists of >5.3% of nuclei in a histogram from more than 600 nuclei collected.*

In a nested case control study the accuracy of these definitions was tested. Residual aneuploidy measured by image cytometry following apparent successful HGD ablation with photodynamic therapy predicted future relapse to HGD or cancer in Barrett's Oesophagus.

**Current limitations of these image cytometry studies:** The correlation with flow cytometry, the current gold standard, was only a small study of ten blinded patients. A more accurate larger

study would need to be performed to truly demonstrate that the findings correlate very closely. This may prove technically extremely difficult because the reference laboratory needs to use fresh frozen tissue and is located in Seattle. A different solution might be to perform a larger case-control or cohort study in a Barrett's surveillance population in the UK using image cytometry. One significant advantage of the ICM technique described is that archive specimens from a cohort could be processed blindly and the true predictive ability ascertained.

The second limitation is that aneuploidy estimation is limited to biopsy samples at present. A cheaper and possibly more accurate method might be to estimate aneuploidy and tetraploidy from a single cytology specimen collected from a Barrett's segment. Cytology has the advantage that a far greater area of the mucosa can be examined than with biopsies alone and in some circumstances endoscopy may not be necessary to collect the specimens. There are however many technical barriers to this including specimen preservation to the laboratory, a new methodology for nuclei extraction and probably new definitions for abnormality of the histograms predicting future cancer risk.

### ***9.1.2 ESS in surveillance***

**The results in this thesis:** This work has demonstrated that ESS may be able to more accurately define a high risk group in Barrett's Oesophagus and perhaps more importantly identify those patients (up to 87%) that do not require further surveillance for at least 5 years. The data in this thesis show that ESS can accurately detect and exclude incorrectly collected spectra from squamous mucosa and detected both HGD and aneuploidy in both algorithm generation and prospective testing. The negative predictive value for an entirely negative scan of a patient's Barrett's Oesophagus has been estimated at over 99% for both HGD and aneuploidy. This may reduce the number of biopsies collected from patients without either abnormality by 60% for HGD and 82% for aneuploidy.

In summary, therefore, the advantages of ESS are that it is easily usable in a DGH with little or no inter-observer variability, it is cheap, quick to assess a Barrett's segment, compatible with existing equipment, requires no extended specialist training and may significantly improve risk stratification.

**Current limitations of these ESS studies:** The limitations are that ESS only reduces but does not eliminate the need for biopsies to be collected in the vast majority of cases with only an estimated 27% of a low risk surveillance population escaping with no biopsies necessary. Secondly, and perhaps most notably, this is really only proof of concept work to date. The next and critical stage of testing is the immediate targeting of high risk areas identified within the endoscopy suite and processing those biopsies first for HGD and then if HGD is absent processing them for aneuploidy. These results should then be compared to standard quadrantic biopsies every 2cm and a posterior series processed for aneuploidy. The software and initial statistical algorithms are now in place for this study to begin although further advanced statistical improvements may be possible with multi-way single step analysis.

### ***9.1.3 Ablative therapies***

**The results in this thesis:** The more widely disputed parameters of ALA PDT, namely drug and light doses and wavelength of activating light, for the treatment of HGD have been resolved by the work presented here. The results have shown that 60mg/kg of ALA activated by at least 1132J/cm of red laser light are most effective. This led to the design and implementation of a partially blinded randomised controlled trial of ALA PDT compared to Photofrin PDT. The latter was chosen as the control arm because at the time of inception it was the minimally invasive therapy of choice for the treatment of HGD in Barrett's Oesophagus. Recruitment has progressed steadily and the study is likely to finish recruiting in June 2008. Already the safety profile with respect to oesophageal stricture formation and number of photosensitivity reactions are divergent between the two groups in favour of ALA PDT and have just reached statistical significance at the 5% level in a one sided Fishers Exact test. The study needs to be completed but the side effect profiles at 4months follow up are fairly robust with most if not all strictures post-Photofrin PDT occurring within 8 weeks of therapy. There also appears to be an advantage in the early eradication rates of HGD for ALA PDT which while statistically significant in a Fisher's Exact Test must be interpreted with caution because the follow up is short and there are only 27 patients at present that have reached 4 months follow up.

**Current limitations of these PDT studies:** The fluence rate for energy delivery might be one further parameter for study with regard to ALA PDT. The relationship between fluence rate and the rate of oxygen free radical production measured by photobleaching is complex and has been shown to influence consequent tissue necrosis (Boere et al. 2006). It is possible that further

improvements to the effectiveness of ALA-PDT or, more likely, the length of the procedure could be made by the monitoring of the tissue kinetics of the photobleaching of PPIX in vivo during treatment.

Secondly, the randomised controlled trial of ALA against Photofrin PDT has not yet been completed. The data, while encouraging in favour of ALA, is on a small number of patients with short follow up and the full results with a median of 3 years follow up are still around 3 years away.

## **9.2 The Future**

A multifaceted approach to tackle the significant outstanding problems regarding Barrett's Oesophagus is almost certainly going to be required.

### ***9.2.1 Primary Screening for Barrett's Oesophagus***

From the perspective of the health of the nation the only plausible way to reduce the overall oesophageal adenocarcinoma rate in the UK is the introduction of an effective primary screening program with the subsequent identification of those at high risk and then offering these patients an effective low risk treatment. It might seem, at present, an entirely unrealistic hope as unlike with colorectal cancer there is no simple but effective and highly specific screening test (eg faecal occult blood testing for colorectal cancer screening). There is then no easy way to detect those at high risk unlike polyp detection at colonoscopy and no widespread, cheap, low risk therapies similar to polypectomy. The work in this thesis addresses at least partially the latter two problems and these are discussed below. Potentially the biggest single obstacle, however, preventing the consideration of screening is the absence of a convenient, relatively accurate, cheap and acceptable investigation.

This screening test for Barrett's Oesophagus would almost certainly have to be non-endoscopic not just on cost grounds but also with respect to endoscopist time and patient acceptability. There have been two interesting but unsuccessful attempts at this non-endoscopic screening with either capsule endoscopy (Sharma et al. 2007) or sponge immunocytochemistry (Lao-Sirieix et al. 2007). In each case while the concepts are ingenious the accuracy of the screening method has been too poor. With regard to the capsule identification of Barrett's oesophagus if the process

could be automated this might improve sensitivity and specificity and reduce costs. For sponge immunocytochemistry could more accurate biomarkers of Barrett's be available or a panel of biomarkers used to possibly even predict subsequent cancer risk? These breakthroughs are seemingly a relatively long way off.

### 9.2.2 Surveillance of Barrett's Oesophagus

Examination of the evidence available suggests that the current format of surveying all patients with Barrett's with quadrantic biopsies every 2cm with the frequency based on histology alone is not viable. Barrett's oesophagus is an easy starting place to identify those that might need surveillance but this has to be targeted at a high risk population. The detection of aneuploidy might offer a solution to this problem. It is estimated that 87% of patients do not have aneuploidy in a surveillance population. The 5 year cancer risk of these patients is very low which from the perspective of health economics is crucial to the cost effectiveness of any surveillance program. The surveillance program might be made more cost effective if follow up was deferred for 6, 7 or even 10 years provided health economists felt it was 'acceptable' to allow a small proportion of cancers to develop. Table 9.1 displays the cancer risk of patients without HGD or aneuploidy in relation to the time from initial baseline endoscopy.

**Table 9.1:** Cancer risk depending on time since negative endoscopy for HGD and Aneuploidy

Follow up from baseline endoscopy	5 years	6 years	7years	10 years
Estimated Cancer Risk if no HGD and no aneuploidy	0%	1.6%	3.6%	6%

(Rabinovitch et al. 2001)

Correctly excluding those at low risk is important but ultimately surveillance is about identifying those patients who will go on to get cancer. While aneuploidy, in combination with HGD, offers a distinct improvement it does not predict exactly who will go on to get cancer. Only 28% of patients with aneuploidy but no HGD will develop cancer within 5 years and therefore 3/4 patients will still need to be surveyed or treated needlessly. For more accurate cancer prediction it is possible that a panel of biomarkers would be required but the likelihood is that some patients who will develop cancer are then missed. As minimally invasive ablative therapies continue to improve it may be that the whole of this group might be treated in the future.

### ***9.2.3 Ablative Therapy***

The future of oesophagectomy for precancerous lesions in Barrett's Oesophagus with all of its associated risks is limited particularly because many of these patients are elderly. At present the use of ablative therapies is confined to patients with HGD mainly due to cost, safety, possible recurrence and the need for ongoing follow up post-ablation. Even the simplest of these techniques, radiofrequency ablation, which is possible to use as an outpatient, would appear not to be cost effective for the ablation of non-dysplastic BO. The NICE guidance in this area, published in December 2007, did not recommend the ablation of non-dysplastic BO and ruled that there is not sufficient evidence for the ablation of HGD in BO.

From the perspective of future studies a randomised controlled trial of oesophagectomy versus photodynamic therapy, a study I felt was the natural next step for several years, I now think is unlikely to occur. Apart from the purely practical issues of finding committed surgeons and gastroenterologists I think it would be difficult to persuade enough patients to undergo surgery in the face of an effective much lower risk alternative. A randomised controlled trial of photodynamic therapy compared to radiofrequency ablation for HGD in Barrett's oesophagus seems the most likely next step with emphasis on safety, patient acceptability, detailed recording of all treatment costs and long term effectiveness.

The last three years have demonstrated the rapid advance of endoscopic technology not just in diagnostic endoscopy but also in minimally invasive endoscopic therapy. The next five years will be an exciting time in upper GI therapeutics and although at present the future appears to be dominated by PDT and radiofrequency ablation I am sure the landscape will shift again to cyrotherapy or some as yet unreported technique further improving patient care.



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